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NOV 17 1938

VOLUME 28 NUMBER 11

NOVEMBER, 1938

Phytopathology

An International Journal

Official Organ of

The American Phytopathological Society

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Price { $6.00 per year, United States and dependencies,
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PUBLISHED MONTHLY BY THE SOCIETY
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THE RELATION OF NITROGEN NUTRITION TO VIRULENCE IN PHYTOMONAS STEWARTI

GEORGE L. MCNEW

(Accepted for publication July 25, 1938)

Although the pathological relationships and physiological characteristics of *Phytomonas stewarti* (E.F.S.) Bergey *et al.* have been determined by extensive studies, very little is known about the physiological processes that enable the bacteria to invade tracheal tubes of maize and cause wilting. Some of the strains of this bacterium, isolated from infested soil (4), infected plants (2, 3, 13), and pure cultures (8) have been reported to differ in virulence. Several such strains were tested recently in order to gain information on the physiological characteristics associated with virulence. It was found that, while the strains were similar in most respects, some of them differed in their nitrogen nutrition. Data on these similarities and differences and on the possible relationship between virulence and nitrogen nutrition are presented in this paper.

MATERIALS AND METHODS

The virulence of cultures was determined by inoculating sets of 15 to 20 sweet-corn seedlings of the variety Golden Bantam with bacteria from a 2- to 3-day-old broth subculture and observing the severity of infection about 10 days later. Differences in virulence are expressed by an infection index, calculated as the average number of necrotic lesions per leaf. The method for determining this index has been described in detail elsewhere (8).

The source and relative virulence at the time of isolation of the strains of *Phytomonas stewarti* used in these studies were as follows:

B–11. A virulent culture isolated from a naturally-infected Golden Bantam corn plant (8). This isolate produced many necrotic lesions and killed some of the invaded leaves. Index = 0.85.

B–1611. An avirulent single-colony isolate obtained from culture B–11. This strain never invaded the leaves sufficiently to cause either yellowing or necrosis of the veins. Index = 0.00.

B–1211. A slightly virulent single-colony isolate obtained from culture B–11. It invaded very few leaves of 10-day-old plants and rarely produced lesions. Index = 0.04.

B–1111. A slightly virulent single-colony isolate obtained from culture B–11. This strain was similar to B–1211, but it invaded a few more leaf veins. Index = 0.11.

B–1411. A virulent strain obtained from B–1111 by a series of single-colony isolations and selection of the most virulent isolate present. It produced many necrotic lesions but wilted very few leaves. Index = 0.64.

B–1011. A highly virulent single-colony isolate obtained from culture B–11

at the same time as B–1211. This strain produced large, necrotic lesions and killed many of the invaded leaves. Index = 1.20.

B–211. A weakly virulent single-colony isolate obtained from a Golden Bantam corn plant inoculated with B–1011. The plant was grown in washed sand in a greenhouse and supplied with a nutrient solution deficient in nitrogen (12). This isolate caused a few, small necrotic lesions but did not wilt the leaves. Index = 0.41.

B–311. A highly virulent strain obtained under the same conditions as B–211, except that the host had received nitrogen at a concentration optimum for plant growth (12). This isolate produced large necrotic lesions and killed about half of the invaded leaves. Index = 1.62.

B–92. A weakly virulent culture isolated by Wellhausen (13) from a wilt-susceptible inbred line of maize designated as GB. This strain invaded the leaves and produced lesions but did not cause wilting. Index = 0.67.

B–91. A highly virulent culture isolated by Wellhausen from a resistant inbred line of maize designated as OSF. It produced large necrotic lesions and killed many of the invaded leaves. Index = 1.76. B–91 and B–92 developed from the same parent culture.

The strains were maintained on nutrient-dextrose agar slants at 8° C. for a year and a half. Although the cultures were transferred to fresh media every 2 or 3 months, none of them changed appreciably in virulence, as was shown by inoculation tests (Table 1) conducted at the termination

TABLE 1.—*The average number of necrotic lesions per leaf and green weights of sweet-corn seedlings inoculated with 10 strains of Phytomonas stewarti*

Strain tested	No. of lesions per leaf						Average green weight per plant gm.
	Replication number					Average	
	1	2	3	4	5		
B–1611	.00	.00	.00	.00	.00	.00 ± .00[a]	14.7 ± 1.0[a]
B–1211	.06	.04	.04	.01	.08	.05 ± .02	14.0 ± 1.7
B–1111	.13	.14	.16	.10	.10	.12 ± .03	13.5 ± 3.2
B–1411	.70	.74	.57	.71	1.06	.76 ± .18	9.1 ± 1.0
B–11	.77	.85	.80	.86	.99	.85 ± .08	12.3 ± 1.7
B–1011	.93	1.12	1.26	1.06	1.22	1.12 ± .15	11.4 ± 2.5
B–211	.52	.42	.64	.49	.49	.51 ± .08	13.7 ± 1.6
B–311	1.24	1.37	1.67	1.41	1.49	1.44 ± .16	8.2 ± 1.6
B–92	.54	.59	.72	.79	.57	.64 ± .11	12.5 ± 1.6
B–91	1.47	1.50	1.87	1.78	1.53	1.63 ± .19	6.3 ± 1.5
Uninoculated controls	.00	.00	.00	.00	.00	.00 ± .00	16.3 ± 1.7

[a] Standard deviation of the mean.

of the experiments to be reported. The data presented in table 1 show that the infection index is reliable to within about .05 of a lesion in determining differences between slightly virulent strains, but only to within about .30 of a lesion for the more virulent strains.

Some of the strains were analyzed for variants. The dilution-plate method was used for separating the variants. It has been shown (9) that practically all of the colonies of *Phytomonas stewarti* in properly prepared dilution plates develop from isolated, single cells. The cultural characteristics of the strains were determined according to the methods outlined by the Committee of the Society of American Bacteriologists (11), except where otherwise noted. Hydrogen-ion determinations were made by the colorimetric method.

The standard synthetic medium used to determine the ability of strains to assimilate inorganic nitrogen contained 0.858 g. KH_2PO_4, 0.684 g. $Ca(NO_3)_2 \cdot 4H_2O$, 1.75 g. $MgSO_4 \cdot 7H_2O$, 0.37 g. $(NH_4)_2SO_4$, 15 g. dextrose, and 15 g. agar per liter of distilled water. For determining the ability to utilize different types of inorganic nitrogen, a nitrogen-deficient medium containing 0.858 g. KH_2PO_4, 1.75 g. $MgSO_4 \cdot 7H_2O$, 0.426 g. $CaCl_2 \cdot 2H_2O$, 15 g. dextrose, and 15 g. agar per liter was supplemented with either $(NH_4)_2SO_4$, $NaNO_3$, or NH_4NO_3 at concentrations sufficient to add 1 mg. of nitrogen per cc. of solution. The salts and sugars were dissolved separately, added to melted agar, adjusted to pH 6.8 to 7.0, and autoclaved for 15 minutes at 20 pounds, pressure. Some of the media were supplemented with amino acids which were obtained from the Eastman Kodak Co. except where otherwise noted.

Tests for physiological characteristics of the strains were made in duplicate and repeated at least once. Inoculation tests were repeated except for those of the single-colony isolates reported in figure 3, A and B. In these 2 experiments a few of the most virulent and least virulent isolates in each group were tested a second time. The results obtained agreed closely with those reported.

Seedlings grown in washed sand and supplied with nutrient solutions according to the method of Spencer and McNew (12) were used in studies on the relation of nitrite production to wilting. The nitrogen-deficient solution described in table 1 of their paper was supplemented with either $(NH_4)_2SO_4$, $NaNO_3$, or NH_4NO_3 at a concentration equivalent to 1 mg. of nitrogen per cc. of nutrient solution.

EXPERIMENTAL RESULTS

Physiological Properties

The 10 strains of *Phytomonas stewarti* possessed the general characteristics attributed to this species (1), with the exceptions described below. All were small, Gram-negative, non-sporogenous, non-motile rods. None produced indol from tryptophane (Difco) or gas in nutrient broth containing

TABLE 2.—*Final pH produced by 7 strains of Phytomonas stewarti after 12 days' incubation at 26° C. in broth containing different carbohydrates*

Carbohydrate in media	pH of media inoculated with culture														pH of uninoculated media	
	B–1211		B–1111		B–1411		B–11		B–1011		B–91		B–92			
Maltose	6.9	6.9	6.9	6.9	6.9	6.8	7.1	7.1	7.1	7.1	7.1	7.1	7.0	7.0	7.0	7.0
Sucrose	5.5	5.7	5.5	5.5	5.5	5.7	5.7	5.7	5.3	5.5	5.5	5.5	5.5	5.5	6.9	6.9
Levulose	5.7	5.7	5.6	5.6	5.6	5.6	5.7	5.7	5.6	5.6	5.6	5.6	5.5	5.5	6.5	6.5
Dextrose	5.3	5.5	5.7	5.5	5.5	5.3	5.3	5.3	5.3	5.4	5.3	5.3	5.3	5.5	6.8	6.8
Mannose	5.7	5.7	5.6	5.6	5.7	5.7	5.7	5.7	5.6	5.6	5.6	5.6	5.6	5.5	6.9	6.9
Arabinose	5.6	5.6	5.6	5.6	5.5	5.5	5.6	5.7	5.6	5.6	5.5	5.5	5.5	5.6	6.2	6.2
Xylose	5.7	5.7	5.6	5.6	5.5	5.5	5.7	5.7	5.7	5.6	5.5	5.5	5.5	5.5	6.2	6.2
Rhamnose	7.1	7.3	7.2	7.3	7.2	7.2	7.3	7.3	7.3	7.3	7.3	7.3	7.2	7.2	6.7	6.7
Dulcitol	8.4	8.4	8.4	8.4	8.2	8.3	8.4	8.4	8.3	8.3	8.2	8.2	8.3	8.2	7.0	7.0
Mannitol	6.3	6.3	6.0	5.9	5.9	5.9	6.2	6.2	6.3	6.3	6.1	6.1	6.2	6.1	6.9	6.9

1 per cent dextrose, levulose, sucrose, maltose, mannose, rhamnose, arabinose, xylose, dulcitol, or mannitol. They produced similar changes in the hydrogen-ion concentration of nutrient broth containing different carbohydrates (Table 2). An acid reaction was induced in broth containing sucrose, levulose, dextrose, mannose, arabinose, xylose, and mannitol; no change was produced in maltose medium, and an alkaline reaction was produced in dulcitol and rhamnose media. B–92 and B–211 produced a firm, dark yellow type of growth in nutrient-dextrose agar poured plates. The other cultures produced smooth, spreading, pale yellow colonies. All strains produced a spreading, liquid, yellow growth on potato slants and caused a slight brown discoloration after 5 days. All produced a yellow sediment in litmus milk (Difco). In addition, B–91 produced acid and curd and finally dissolved the curd.

The virulent strains grew readily, on the standard synthetic medium, while the slightly virulent B–1211 and B–1111 failed to produce visible growth. During subsequent tests, it was observed that these 2 cultures may rarely produce small isolated colonies along the inoculation streak. As a general rule, however, the slightly virulent strains failed to grow either on the standard or other synthetic media (Fig. 1). The avirulent B–1611 grew as readily on the synthetic media as the virulent strains. Of the cultures that grew on the standard medium, B–91 was the only one that produced nitrites.

The standard synthetic medium was supplemented with several organic materials in an attempt to determine what nutrients were lacking for the slightly virulent strains B–1211 and B–1111. The standard medium without dextrose was supplemented with 1 per cent (except as otherwise noted) dextrose, sucrose, glycerine (5 per cent), mannitol, starch, dextrin, salicin, lactose, nutrient broth (Difco, 0.8 per cent), peptone (Fairchild's), or tryptophane. The synthetic medium with dextrose was supplemented with 1 per cent peptone, *l*-glutamic acid, *d, l*-phenylalanine (Pfanstiehl), aspartic acid, tyrosine, glycine, *d, l*-alanine, *d, l*-valine, egg yolk, egg albumen, casein, gelatine, urea, or asparagine (Eimer and Amend). The 2 slightly virulent strains failed to grow on any of these media, except those containing organic nitrogenous supplements (Fig. 2). Most of the amino acids proved to be less nutritive than peptone, egg yolk, or nutrient extract (Table 3). The virulent strains grew on all media except those containing only starch, dextrin, salicin, or lactose as a carbohydrate source. These strains were inhibited by phenylalanine, alanine, and valine, and slightly inhibited by urea at the concentration used.

The data show that, although the strains were similar in most respects, they differed in their ability to assimilate nitrogen. Two of the less virulent strains assimilated only nitrogen from organic sources, the strains of intermediate virulence assimilated nitrogen from inorganic salts without reducing nitrates, and the most virulent strains tested reduced nitrates to nitrites.

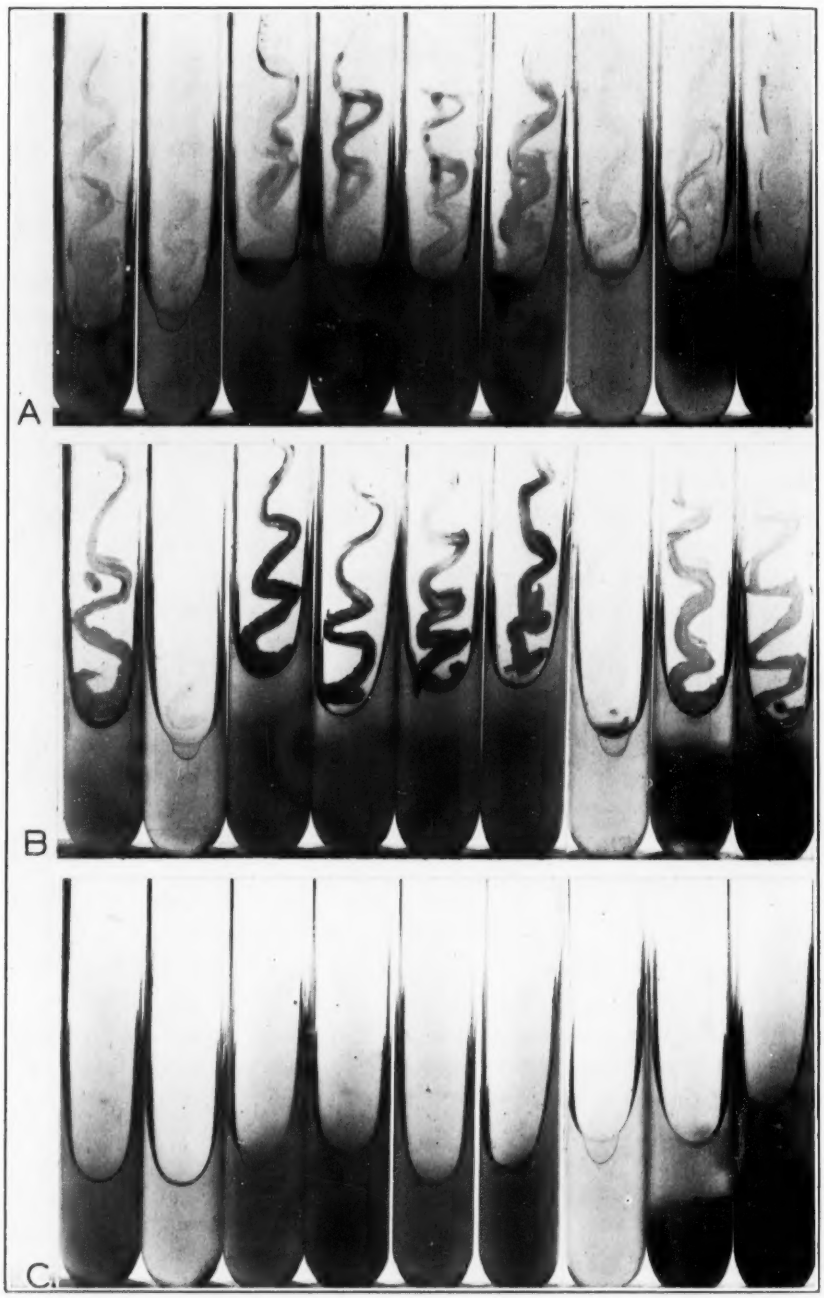

FIG. 1. Growth of highly virulent strain B–1011 (A), weakly virulent B–211 (B), and slightly virulent B–1211 (C), on different synthetic media. From left to right, the tubes contain dextrose agar with the following salt solutions: Standard medium; nitrogen-deficient medium supplemented with sufficient NH_4NO_3 to add 0.0, 0.5, 1.0, 2.0, and 4.0 mg. of nitrogen per cubic centimeter; and phosphorus-deficient solution (.558 g. K_2SO_4, .684 g. $Ca(NO_3)_2 \cdot 4H_2O$, 1.75 g. $MgSO_4 \cdot 7H_2O$, .370 g. $(NH_4)_2SO_4$ per liter) supplemented with sufficient NaH_2PO_4 to add 0.0, 0.5, and 1.0 mg. of phosphorus per cubic centimeter, respectively. The slightly virulent strain grew poorly, if at all, on all of these media, while the more virulent strains grew readily on all except those deficient in nitrogen and phosphorus. (Photographs by J. A. Carlile.)

TABLE 3.—*Growth of 7 strains of Phytomonas stewarti on synthetic agar medium with and without organic supplements*

Organic supplement		Relative development of different strains[a]						
Nitrogen	Carbohydrate	B–1211	B–1111	B–1411	B–11	B–1011	B–91	B–92
None	Dextrose	–, –	–, –	+, +	++, ++	++, ++	++, ++	++, ++
"	Sucrose	–, –	–, –	+, tr	++, ++	++, ++	++, ++	++, ++
"	Glycerine	–, –	–, –	+, +	++, ++	++, ++	++, ++	++, ++
"	Mannitol	–, –	–, –	+, +	++, ++	++, ++	++, ++	++, ++
"	Starch	–, –	–, –	–, –	–, –	–, –	–, –	–, –
"	Dextrin	–, –	–, –	–, –	–, tr	tr, tr	–, –	tr, tr
"	Salicin	–, –	–, –	–, –	–, –	–, –	–, –	–, –
"	Lactose	–, –	–, –	–, –	–, –	–, –	–, –	–, –
Nutrient broth	None	++, ++	++, ++	++, ++	++, ++	++, ++	++, ++	++, ++
Peptone	"	++, ++	++, ++	++, ++	++, ++	++, ++	++, ++	++, ++
Tryptophane	"	++, ++	++, ++	++, ++	++, ++	++, ++	++, ++	++, ++
None	"	–, –	–, –	–, –	–, –	–, –	–, –	–, –
Peptone	Dextrose	++, ++	++, ++	++, ++	++, ++	++, ++	++, ++	++, ++
Glutamic acid	"	–, –	–, –	+, +	++, ++	++, ++	++, ++	++, ++
Phenylalanine	"	–, –	–, –	–, –	–, –	–, –	–, –	–, –
Aspartic acid	"	–, –	–, –	+, +	++, ++	++, ++	++, ++	++, ++
Tyrosine	"	+, –	+, tr	+, +	++, ++	++, ++	++, ++	++, ++
Glycine	"	–, –	–, –	+, +	++, ++	++, ++	++, ++	++, ++
Alanine	"	–, –	–, –	–, –	–, –	–, tr	++, ++	–, +
Valine	"	–, –	–, –	+, +	+, +	+, –	+, +	+, +
Egg yolk	"	++, ++	++, ++	++, ++	++, ++	++, ++	++, ++	++, ++
Egg albumen	"	+, +	tr, tr	+, +	++, ++	++, ++	++, ++	++, ++
Casein	"	tr, tr	+, +	+, +	++, ++	++, ++	++, ++	++, ++
Gelatine	"	+, ++	+, +	+, +	++, ++	++, ++	++, ++	++, ++
Urea	"	–, –	–, –	+, tr	+, +	+, +	++, ++	++, ++
Asparagine	"	–, tr	tr, +	+, +	+, +	++, ++	++, ++	++, ++

[a] –, no growth; tr, isolated spots; +, faint streak; ++, abundant growth.

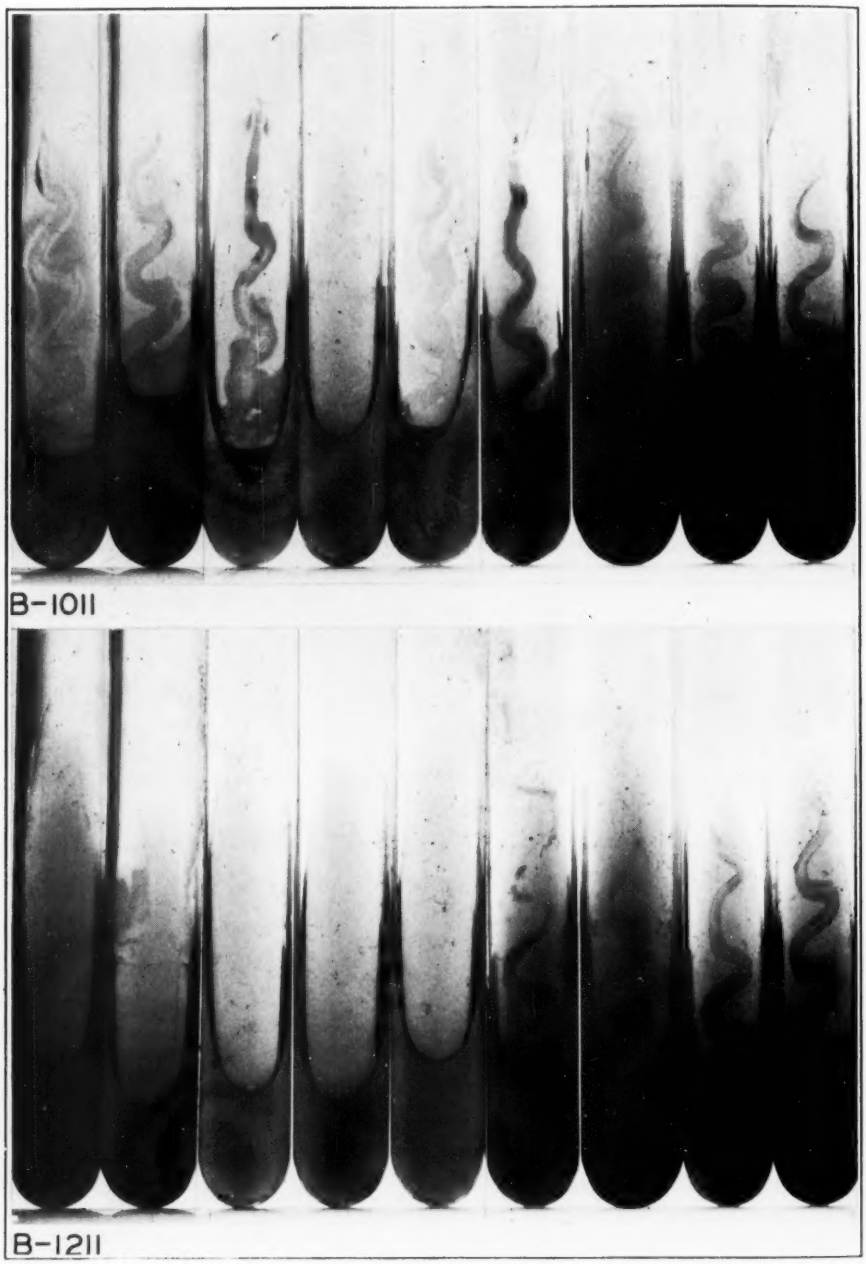

FIG. 2. Growth of the highly virulent strain B–1011 and the slightly virulent B–1211 on synthetic agar containing different organic supplements. From left to right, the tubes contain as supplements to the salt solutions: Dextrose, sucrose, glycerine, lactose, mannitol, dextrose and peptone, nutrient broth, peptone, and tryptophane. The slightly virulent culture grew only on those media that contained organic nitrogen. (Photographs by J. A. Carlile.)

Correlation of Invasiveness with Ability to Assimilate Inorganic Nitrogen

Since *Phytomonas stewarti* is a vascular parasite, it probably secures its nutrients from the transpiration stream. It seemed possible, therefore, that the differences in virulence between strains might be explained by differences in their ability to assimilate inorganic nitrogen. For this reason, experiments were made to determine whether *P. stewarti* must be capable of using inorganic nitrogen before it can invade maize and whether the nitrites produced by some strains were responsible for the severe wilting observed.

The possibility that cultures must be able to use inorganic nitrogen before they can invade the tracheal tubes was tested first. Experiments were designed to determine (1) whether all virulent cultures use inorganic nitrogen, (2) whether slightly virulent cultures regain the ability to use inorganic nitrogen when they are restored to full virulence by host passage, and (3) whether slightly virulent cultures become more virulent as they regain the ability to use inorganic nitrogen.

In the first experiment, 757 single-colony isolates from the virulent strains described in this paper and elsewhere (8) were tested for virulence and ability to use inorganic nitrogen. All of the virulent and highly virulent and some of the slightly virulent isolates grew on the synthetic medium. Only one culture failed to grow; it was found to be slightly virulent. These observations suggested that the ability to utilize inorganic nitrogen is characteristic of virulent strains.

The procedure in the second experiment was to restore culture B–1211 to virulence by host passage and then to test single-colony isolates from both the original culture and the inoculated plants for ability to grow on synthetic media. A sample from a broth subculture of B–1211 was mixed with melted agar for dilution plates before the remainder was injected into 30-day-old plants. Forty-two days after inoculation, tissues from a small, yellow leaf lesion and from the stems of 3 systemically invaded plants were macerated, diluted in broth, and suspended in melted agar for dilution plates. Fifty single-colony isolates from the broth subculture and 40 from each of the 3 plant tissues were tested for virulence. The data obtained (Fig. 3, A) show that the subculture of B–1211 contained variants, but that none of them had an index of more than 0.20. The isolates recovered from the leaf lesion were only slightly more virulent than the bacteria injected. Most of those recovered from the stems were fully virulent. They had indexes ranging from 0.10 to 1.30, with most of them between 0.40 and 1.00. All of the isolates recovered from the broth culture and the leaf lesion grew poorly on the synthetic medium. The virulent isolates recovered from the stems, however, grew very readily on this medium; the entire streak was covered with bacteria within 48 hours and masses of bacteria usually collected at the base of the slants (Fig. 1). Isolates from the stem that had indexes of less than 0.45 grew poorly on the synthetic medium.

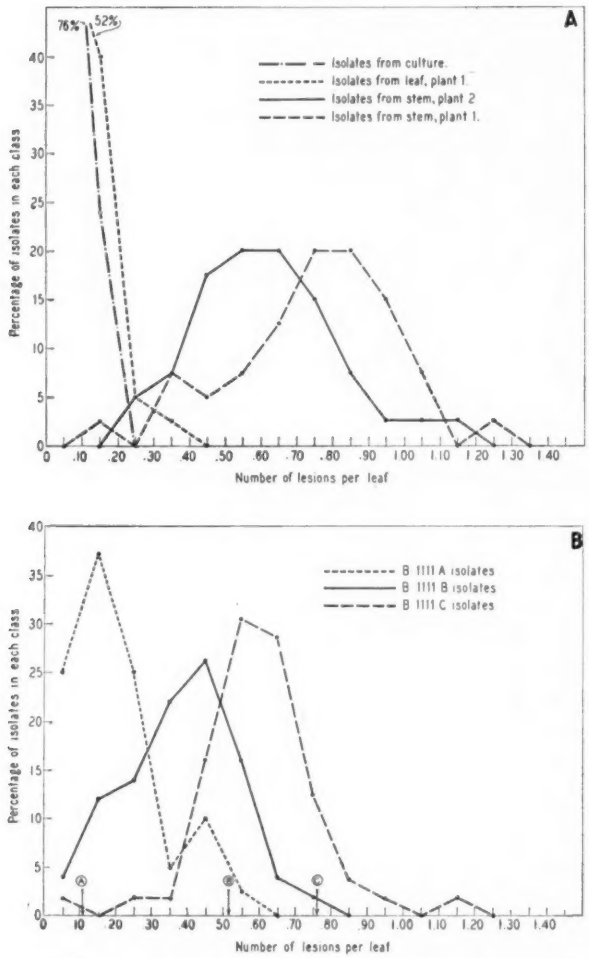

FIG. 3. Distribution of single-colony isolates from slightly virulent strains of *Phytomonas stewarti* in pathogenic classes that differ by 0.10 of a lesion each. A. The isolates recovered from the stems of sweet-corn plants inoculated by strain B–1211 were much more virulent than those obtained from either an invaded leaf vein or the culture inoculated into the plants. B. Virulent strains were obtained from culture B–1111 by pouring a series of dilution plates and selecting the most virulent isolate present. This isolate was used as inoculum for the next dilution. Isolates obtained from the third selection (B–1111 C, index of 0.76) had infection indexes that ranged from 0.00 to 1.20.

In the third experiment, cultures B–1211 and B–1111 were induced to grow on the synthetic medium and then tested for virulence. In a typical test, culture B–1211 was transferred to 80 slants of synthetic agar and several of nutrient agar. Isolated points of growth occurred on some of the synthetic slants after 5 or 6 days. Successful transfers to other synthetic agar slants were secured from only 3 of these isolated colonies. After 3 serial transfers, the cultures on synthetic agar and those on nutrient agar were transferred to tubes of nutrient-dextrose broth, incubated for 18 hours, and then tested for virulence. The data presented in table 4 show that, without exception, the

TABLE 4.—*Virulence of culture B–1211 for sweet-corn seedlings after passage through nutrient and synthetic media*

Test No.	Treatment of culture		No. of plants tested	Status of leaves			Infection index
	Media used	No. of passages		Total no.	No. wilted	No. of lesions	
I	Nutrient	3	15	75	0	1	.01
	"	3	15	77	0	2	.02
	"	3	13	70	0	1	.01
	Synthetic	3	15	76	7	39	.79
	"	3	12	58	7	34	.95
	"	3	14	65	1	42	.69
	Uninoculated controls		15	83	0	0	.00
II	Nutrient	10	15	102	0	7	.07
	Synthetic	10	15	104	1	34	.36
	"	10	15	105	2	74	.76
	"	10	15	110	0	62	.56
	Uninoculated controls		15	113	0	0	.00

cultures incubated on synthetic agar were more virulent than those on nutrient agar. Comparable results were secured in similar tests with culture B–1111 (Fig. 4).

The foregoing data show that the ability of *Phytomonas stewarti* to use inorganic nitrogen is closely correlated with virulence. All of the virulent cultures were able to use inorganic nitrogen readily, whereas the less virulent ones were not. When slightly virulent cultures were restored to full virulence either by host passage or by incubation on the synthetic medium, they regained the ability to assimilate inorganic nitrogen. The results suggest that virulence is dependent upon the ability to use inorganic nitrogen and support the hypothesis that *P. stewarti* must use inorganic nitrogen before it can secure sufficient nutrients from the transpiration stream to be fully invasive.

Selection of Virulent Strains from Slightly Virulent Cultures

The observations reported above show that the attenuated cultures were restored to virulence by incubation in the host or on the synthetic medium containing no organic nitrogen. The data offer little evidence, however, of the mechanism by which this change was brought about. An experiment was performed, therefore, to determine whether the more virulent strains were produced by a direct physiological stimulus on the individual, slightly virulent cells or merely by a differential selection of variant types by the host and the medium.

Strain B–1111, cultured on nutrient agar, was incubated in nutrient broth and then suspended in melted agar for dilution plates. Forty single-colony isolates were found to differ widely in virulence. Under the conditions

FIG. 4. Sweet-corn plants inoculated with culture B–1111 from nutrient-dextrose agar slants (A) and from synthetic agar slants (B). The 2 cultures were transferred twice on their respective media, subcultured in nutrient-dextrose broth for 18 hours, then injected into 10-day-old plants. The photographs were taken 20 days after inoculation. (Photographs by J. A. Carlile.)

of this experiment, culture B-1111 had an index of 0.11 and its most virulent strain (B–1111B) an index of 0.52 (Fig. 3, B). This strain, B–1111B, was maintained on nutrient agar slants for 28 days and then tested for variants. One of the 40 single-colony isolates obtained had an index of 0.76 (B–1111C). This isolate was, in turn, tested for variants after it had grown on nutrient agar slants for 30 days. The data presented in figure 3, B, show that 56 single-colony isolates obtained from B–1111C had indexes ranging from 0.10 to 1.20. Most of the isolates were similar to the parent, since they had indexes of 0.40 and 0.80. This experiment shows that it is possible to secure virulent cultures from slightly virulent ones by selecting the most virulent strains that are produced on an ordinary culture medium. The data also show that the restoration of virulence to the attenuated culture occurred by a number of definite steps; that is, a slightly virulent cell produced a weakly virulent one, the weakly virulent one produced a virulent one, and the virulent one finally produced others that were even more virulent. There was evidence that the new variants tended to produce many cells that reverted to the original slightly virulent parent type (B–1111).

The 56 isolates obtained from the plate poured with B–1111C were tested on a synthetic medium. All of those having infection indexes of more than 0.70 grew readily, and those with indexes of less than 0.45 grew poorly if at all. Some of the isolates of intermediate virulence (index 0.45 to 0.70) grew, while others failed to develop visible growth. Although the virulent isolates obtained from nutrient media always grew when transferred to the synthetic medium, they did not grow so readily as virulent cultures recovered from the host. They required about 1 day longer to produce visible growth and about 2 days longer to completely cover the slant than the virulent isolates obtained from the host.

Several of the isolates with indexes of 0.45 to 0.70 that grew poorly on the synthetic medium were subcultured, inoculated into seedlings, and isolated as soon as lesions were produced (4 to 6 days). The isolates grew readily on the synthetic medium. This observation suggests that these strains were invasive because they readily produced progeny that were capable of using inorganic nitrogen.

The above data show that slightly virulent strains produced variants some of which are more virulent than themselves. On ordinary nutrient agar the highly pathogenic variants constitute such a small proportion of the population that the culture, as a whole, is almost avirulent. The culture became more virulent, however, as the variants were selected out and the less virulent strains eliminated. Apparently passage either in the host or on the synthetic medium selected out the virulent types. There is little reason to assume that the bacterial cells were changed by a physiological stimulus of the host or the synthetic medium, since the same change in virulence was effected on nutrient agar by a simple mechanical selection of variants.

The Rôle of Nitrites in Producing Wilt

It having been shown above that there is a close correlation between ability

to use inorganic nitrogen and invasiveness, attention was next directed to the question of whether or not the nitrites produced by some strains (B–91) were responsible for the severe wilting observed. The wilting induced by other vascular parasites, such as species of *Fusarium*, has been attributed (10) to the nitrites they produce. In order to establish such an explanation for wilting, it would be necessary to demonstrate that highly virulent strains produce nitrites, that nitrites are produced in the severely wilted hosts, and that solutions of nitrites cause healthy plants to wilt.

Forty single-colony isolates obtained from B–91 were tested for virulence and ability to reduce nitrates, in order to determine whether or not these properties are correlated. Twelve of the isolates failed to reduce nitrates in culture. Most of these isolates were almost as virulent as those that reduced nitrates. This observation agrees with the data obtained on B–1011 and B–311. Although these 2 strains failed to reduce nitrates in culture, they were almost as virulent as B–91 (Table 1).

The above observation suggested that nitrite production is not essential for high virulence in *Phytomonas stewarti*. Further studies were made on nitrite production because it was observed that some cultures, such as B–1011, that did not produce nitrites in culture caused accumulation of nitrites in completely wilted seedlings. Leaf whorls from 10 15-day-old plants killed by B–1011 all contained nitrites, but positive tests were rarely secured from recently-formed leaf lesions. Both the leaf whorl and small leaf lesions in plants invaded by B–91 contained nitrites. Cultures B–91, B–92, and B–1011 were, therefore, tested further for ability to utilize different types of inorganic nitrogen and to reduce nitrates, in the hope of finding an explanation of why B–1011 produced nitrites in the host but not in culture. These strains were transferred to synthetic media containing either $(NH_4)_2SO_4$, NH_4NO_3 or $NaNO_3$. All the strains grew readily on the 2 media containing ammonium; but, as reported above, B–91 was the only one to reduce NH_4NO_3 to nitrites. No nitrites were produced in the $(NH_4)_2SO_4$ medium. Culture B–91 was the only strain that grew as readily on the $NaNO_3$ medium as on the ammonium-containing ones. Culture B–1011 did not produce visible growth on this medium until 5 days after inoculation, and then it was very feeble. Successful transfers were secured from some of these slants and after 2 passages on the nitrate medium the bacteria were found capable of reducing nitrates. However, such nitrate-reducing strains of B–1011 failed to reduce nitrates when subsequently transferred back to the NH_4NO_3 medium. Culture B–92 failed to grow on the $NaNO_3$ medium, even after long incubation periods. These data show that B–91 readily used either ammonium or nitrate nitrogen but that B–1011 used nitrates only when deprived of the other type of nitrogen. This observation suggests that B–1011 did not reduce nitrates in culture because there was an excess of ammonium salts, but that it was forced to utilize nitrates in the host after the supply of ammonium nitrogen was exhausted. This hypothesis is sup-

ported by the observation that B–1011 had produced no nitrites in recently invaded leaf tissue.

The nitrites produced in the plant by B–91 and B–1011 may have been responsible for some of the symptoms observed. Water-soaked streaks were produced in the leaves of healthy 8-day-old seedlings injected with 2 per cent solutions of either $NaNO_2$ or KNO_2. More concentrated solutions caused a general wilting and collapse of the plants. Solutions of either KNO_3 or $NaNO_3$ at 10 times this concentratin caused no appreciable wilting. Excised 20-day-old plants were severely wilted after 24 hours in 0.5 per cent solutions of either $NaNO_2$ or KNO_2.

Although the foregoing observations show that bacteria produce nitrites within the host and that the symptoms of nitrite toxicity resemble bacterial wilt, they do not definitely establish the causal relationship of nitrite production to wilting. The question arises as to whether the bacterium can cause wilting under conditions that prevent nitrite formation. Evidence was obtained on this question by taking advantage of the fact that the bacterium does not produce nitrites from ammonium and that the contents of the tracheal tubes may be modified by the nutrient treatment supplied to plants (7). Four sets of 30 seedlings each were grown in washed sand supplied with either a nitrogen-deficient solution or with this solution supplemented with either $(NH_4)_2SO_4$, $NaNO_3$, or NH_4NO_3. Half of the plants receiving each of these nutrient treatments were inoculated with culture B–1011. Observations made 15 days after inoculation showed that the plants deprived of nitrogen were less severely wilted than those supplied with nitrogen (Table 5). The plants supplied with ammonium nitrogen

TABLE 5.—*Virulence and nitrite production of Phytomonas stewarti in sweet-corn seedlings receiving different types of nitrogen*

Treatment of plant		No. of plants tested	Status of leaves			Infection index	Total green weight gm.	Nitrite test
Nitrogen source	Inoculation		Total no.	No. wilted	No. of lesions			
NH_4NO_3	B–1011	15	68	32	50	2.15	21.5	+
NH_4NO_3	None	15	86	0	0	.00	90.0	–
$NaNO_3$	B–1011	15	70	22	60	1.80	32.0	+
$NaNO_3$	None	15	91	0	0	.00	108.0	–
$(NH_4)_2SO_4$	B–1011	15	62	27	35	1.87	15.0	–
$(NH_4)_2SO_4$	None	15	78	0	0	.00	86.0	–
None	B–1011	15	71	1	69	1.01	34.0	–
None	None	15	73	0	0	.00	60.0	–

were as severely wilted as those supplied with either nitrate or ammonium and nitrate nitrogen. Wilted plants supplied with nitrates were found to contain nitrites, but those receiving only ammonium were not. The extremely high infection indexes secured with culture B–1011 in this experiment were due to the high nitrogen levels and the poor growing conditions prevailing at the time of these tests (February, 1937). Repetition of this

experiment later in the spring gave comparable results, except that all plants were less severely wilted.

The above data show that *Phytomonas stewarti* may cause wilting without producing nitrites in detectable quantities. The nitrites ordinarily produced in diseased plants grown in soil are toxic and undoubtedly contribute towards the production of wilt symptoms, even though they are not the sole cause of wilting. The aggressiveness of strains such as B–91 that readily use either ammonium or nitrate nitrogen may be due to the fact that more nitrogen is available in the tracheal tubes for their growth than for the strains that are specialized for ammonium nitrogen.

DISCUSSION

It is a matter of common observation that most pathogenic bacteria become attenuated on culture media. The standard method for restoring virulence to such cultures is to pass them through appropriate hosts, but the exact mechanism by which the host changes the culture is not known. The data presented above suggest an explanation of what might occur during the attenuation and restoration of virulence in *Phytomonas stewarti*.

Culture B–11 was 5 times purified by dilution and single-colony isolation (8) in order to insure its single-cell origin. This culture was virulent and capable of using nitrogen from inorganic salts. The evidence obtained indicated that this type of nitrogen nutrition was essential for virulence. After several transfers on nutrient-dextrose media, B–11 produced slightly virulent strains (B–1211 and B–1111) that were incapable of using nitrogen from inorganic materials. These strains grew as well as the more virulent parent type on nutrient-dextrose media. It is possible that in time they might have replaced the parent type and caused attenuation of the culture, just as de Kruif's (5, 6) cultures of the rabbit septicemia bacillus lost virulence as the avirulent form replaced the parent type. This change was brought about artificially by separating B–1211 and B–1111 from the parent type in a poured agar plate.

When cultures B–1211 and B–1111 were injected into the host, they probably failed to grow because of the limited supply of organic nitrogen in the tracheal tubes. They did multiply in a very few leaf veins, but did not spread or cause severe injury (Fig. 3, A). The only bacteria that multiplied extensively, became systemic, and caused large lesions were the progeny of B–1211 that were capable of using inorganic nitrogen. These progeny were apparently of the same type as the variants separated by the dilution-plate method from cultures of B–1111, grown on nutrient-dextrose agar. The fact that this same type of variant could be selected on the synthetic medium offered additional evidence that the host served as a selective medium for those strains capable of using nitrogen from inorganic sources.

The foregoing discussion is based upon the hypothesis that *Phytomonas stewarti* must utilize nitrogen from inorganic sources before it can be invasive. The data presented on the close correlation of these 2 properties may be ac-

cepted as evidence either that they are one and the same thing or that they are independent characteristics that are always inherited together. The latter suggestion would be based upon the assumption that there are genes and linkage phenomena in bacteria as well as in higher plants. Although this possibility is not disputed, it is believed that the data can be explained best upon the assumption that nitrogen nutrition conditions virulence. The ability of slightly virulent strains, such as B–1611, to utilize nitrogen from inorganic sources need not detract from the hypothesis, since the avirulence of such strains may be attributed to some deficiency other than the inability to use inorganic nitrogen. On the other hand, the causal relationship of the 2 properties would be definitely disproved if any highly virulent strain failed to use inorganic nitrogen. Such a strain was not observed in the rather extensive tests conducted.

The data obtained show that an attenuated culture regains virulence by producing a series of variants, each one of which is a little more virulent than its predecessor. With our present knowledge of heredity in bacteria there is no logical explanation of why the evolution occurs in a series of steps rather than by one change from the avirulent to fully virulent. Likewise, there is no explantion of why some of the slightly more virulent variants that are produced show no corresponding increase in ability to use nitrogen from inorganic sources. It is possible that a difference in the nitrogen nutrition of these strains could be detected by employing some other method of measuring the ability to use inorganic nitrogen. The significant point in the present thesis is that cultures restored to full virulence always regained the ability to use inorganic nitrogen.

The foregoing discussion on nitrogen nutrition applies to the early stages of invasion while the bacteria are confined to the tracheal tubes. Since these tubes are composed of dead cells, the question may be raised as to whether *Phytomonas stewarti* may be considered as a true pathogen. There seems to be no legitimate reason for not considering it as such, because the bacteria produce characteristic disease symptoms and, in the later stages of invasion, penetrate the parenchymatous tissues surrounding the leaf veins (4). The invasion of these living tissues probably is controlled by ability to produce enzymes or toxins, but such materials have not yet been identified. In addition to the nitrites produced by some strains (B–91), the bacteria probably produce other materials that injure the host cells.

SUMMARY

Ten strains of *Phytomonas stewarti* that differed in virulence were found to have the following morphological, cultural, and physiological characteristics in common. All consisted of small, Gram-negative, non-motile, non-sporogenous rods. None of them produced indol from tryptophane or gas from carbohydrates. They caused similar changes in the acidity of media containing different carbohydrates and produced the same type of growth on potato slants.

The strains differed from each other in that 2 slightly virulent ones failed to utilize nitrogen from inorganic sources, 2 other strains produced small, firm, yellow colonies on nutrient-dextrose-agar poured plates, and the most virulent strain reduced nitrates to nitrites and produced a curd in litmus milk.

A series of experiments showed that ability to use inorganic nitrogen was correlated always with virulence. All virulent cultures used inorganic nitrogen, and slightly virulent cultures regained the ability when restored to virulence by host passage. Slightly virulent cultures became more virulent after they had been induced to grow on a synthetic agar medium. These changes in virulence and physiological abilities apparently were brought about as the result of an intensive selection of variants that were produced by the slightly virulent culture. The slighly virulent strain produced variants that in turn produced other variants. By proper mechanical separation and selection of these variants on nutrient agar it was possible to derive a virulent culture from a slightly virulent one by a method analogous to that occurring in the host.

Most of the virulent strains utilized ammonium nitrogen much more readily than nitrate nitrogen. The most virulent strain tested (B–91) reduced nitrates to nitrites in a medium containing both ammonium and nitrate nitrogen. This and other virulent strains produced nitrites in severely wilted plants, but the nitrites were found not to be the sole cause of the wilting.

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LITERATURE CITED

1. ELLIOTT, CHARLOTTE. Manual of Bacterial Plant Pathogens. 349 pp. The Williams and Wilkins Co. (Baltimore). 1930.
2. HOLBERT, J. R., CHARLOTTE ELLIOTT, and B. KOEHLER. Bacterial leaf blight of dent corn. (Abstract) Phytopath. **23**: 15–16. 1933.
3. IVANOFF, S. S., A. J. RIKER, and H. A. DETTWILER. Studies on cultural characteristics, physiology and pathogenicity of strain types of *Phytomonas stewarti*. Jour. Bact. **35**: 235–253. 1938.
4. ———. Stewart's wilt disease of corn, with emphasis on the life history of *Phytomonas stewarti* in relation to pathogenesis. Jour. Agr. Res. [U. S.] (1933) **47**: 749–770. 1934.
5. KRUIF, P. H. DE. Dissociation of microbic species. I. Coexistence of individuals of different degrees of virulence in cultures of rabbit septicemia. Jour. Expt. Med. **33**: 773–789. 1921.
6. ———. Virulence and mutation of the bacillus of rabbit septicemia. Jour. Expt. Med. **35**: 621–633. 1922.
7. LOWRY, M. W., W. C. HUGGINS, and L. A. FORREST. The effect of soil treatment on the mineral composition of exuded maize sap at different stages of development. Georgia Agr. Exp. Sta. Bull. 193. 1936.
8. MCNEW, G. L. Isolation of variants from cultures of *Phytomonas stewarti*. Phytopath. **27**: 1161–1170. 1937.
9. ———. Dispersion and growth of bacterial cells suspended in agar. Phytopath. **28**: 387–401. 1938.
10. ROSEN, H. R. Efforts to determine the means by which the cotton-wilt fungus, *Fusarium vasinfectum*, induces wilting. Jour. Agr. Res. [U. S.] (1926) **33**: 1143–1162. 1927.

11. SOCIETY OF AMERICAN BACTERIOLOGISTS. Committee on Bacteriological Technic. Manual of Methods for Pure Culture Study of Bacteria. (Geneva, N. Y.) 1933 and supplements to date.
12. SPENCER, E. L., and G. L. MCNEW. The influence of mineral nutrition on the reaction of sweet-corn seedlings to *Phytomonas stewarti*. Phytopath. **28**: 213–223. 1938.
13. WELLHAUSEN, E. J. Effect of genetic constitution of the host on the virulence of *Phytomonas stewarti*. Phytopath. **27**: 1070–1089. 1937.

VARIABILITY OF GLOMERELLA GOSSYPII

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(Accepted for publication May 5, 1938)

INTRODUCTION

Variations in the cultural behavior of certain isolates of *Glomerella gossypii* (South.) Edg. were observed during an investigation on the organisms involved in damping off of cotton seedlings. These variations appeared in the original isolations, suggesting that such cultural types occur in nature. The purpose of the present paper is to report observations on the variability of this fungus in respect to cultural behavior, and pathogenicity on cotton seedlings.

STUDIES ON VARIATION IN CULTURAL BEHAVIOR

As soon as colonies of *Glomerella gossypii* were recognized in platings of diseased materials, records were made of their growth characters and single conidial isolations of each were made to individual plates of potato-dextrose agar. After the monosporous colonies had grown a few days they were placed at 8°C. for future use. In taking notes on the colonies particular attention was paid to such characters as type of aerial mycelium, color of aerial and subaerial mycelium, type of colony margin, and growth rate of the colony.

After 58 monosporous isolates finally were collected, they were removed from the low-temperature chamber and single-spore transfers of each were made to fresh plates of potato-dextrose agar. These cultures were incubated at 27°C. and, after 6 days, their cultural characters were compared and recorded. The same variant cultures observed in the original platings appeared in the respective single-spore cultures.

Of the 58 isolates 45 were alike, so far as growth behavior was concerned. These were arbitrarily referred to as "normal-type" colonies. The remaining 13 cultures differed from the normal type in one or more characters and were referred to as "variant-type" colonies.

These preliminary observations suggested that more detailed studies of the phenomenon of variation would be of interest. An experiment, therefore, was set up for the purpose of determining the extent, frequency, and magnitude of variation as observed in pure culture and under constant environmental conditions. In the experiment 27 single conidial cultures were used. Fourteen of these were of the normal type (Fig. 1, A)

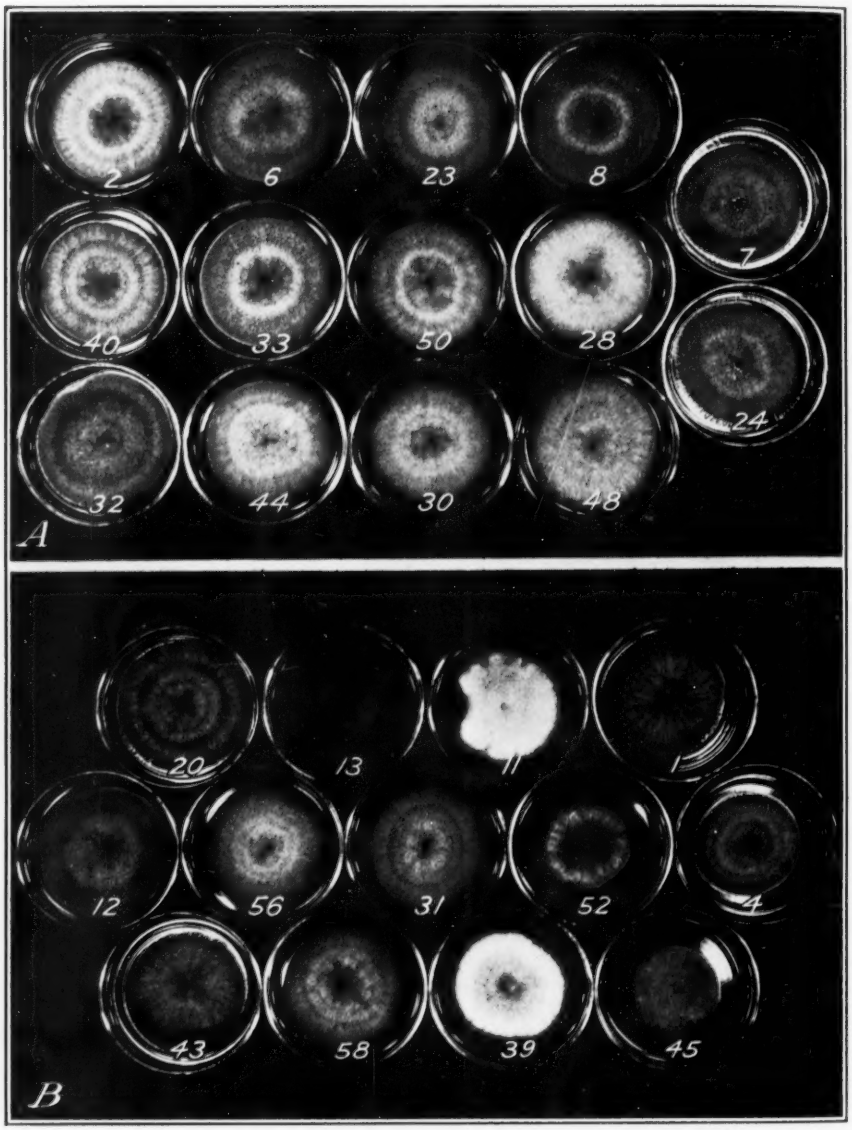

FIG. 1. Original single-conidial isolates employed in the studies on cultural behavior and pathogenicity as they appeared at the beginning of the investigation. A. "Normal-type" isolates. B. "Variant-type" isolates.

and 13 of variant types (Fig. 1, B). The history of these cultures is given in table 1.

The isolates were grown exclusively on potato-dextrose agar,[1] and all

[1] The potato-dextrose agar was made up as follows: 200 grams of peeled potatoes were placed in 500 cc. of distilled water and autoclaved for 20 minutes. The decoction was filtered and 20 grams of dextrose dissolved in it. To this 500 cc. of 4 per cent agar was added and the mixture brought up to 1 liter by the addition of water. The medium was then placed in flasks or tubes and autoclaved 20 minutes.

cultures, either on slants or in Petri plates, were incubated in the dark at 27°C. Cultures always were compared on the same batch of agar, and a uniform quantity of it was used in Petri plates (20 cc.) and slants (5 cc.). The method of handling the cultures in this experiment consisted in making weekly a transfer of each isolate by means of a spore suspension to a fresh slant. On alternate dates of transfer single-spore isolations were made from each culture in triplicate, to 3 plates of agar. When the colonies were 6 days old notes were taken on their cultural behavior and the appearance of any variant types recorded. When any of the 3 colonies isolated from a given culture showed a marked variation from the original it was transferred to a slant and the parent culture discarded.

TABLE 1.—*History of cultures of Glomerella gossypii employed in the study of variability of the causal organisms of damping off of cotton seedlings*

Culture number	Host	Origin of host	Culture number	Host	Origin of host
V[a]–1	cotton seedling	Auburn, Ga.	V–31	cotton seedling	Pendleton, S. C.
N– 2	do.	do.	N–32	do.	Auburn, Ga.
V– 4	do.	do.	N–33	do.	do.
N– 6	do.	do.	V–39	do.	Pendleton, S. C.
N– 7	do.	do.	N–40	do.	Auburn, Ga.
N– 8	do.	do.	V–43	do.	do.
V–11	do.	do.	N–44	do.	do.
V–12	do.	do.	V–45	do.	Kathwood, S. C.
V–13	do.	do.	N–48	do.	Auburn, Ga.
V–20	cotton seed	do.	N–50	do.	Pendleton, S. C.
N–23	cotton seedling	Chester, S. C.	V–52	do.	Virginia
N–24	cotton seed	Auburn, Ga.			
N–28	cotton seedling	Pendleton, S. C.	V–56	boll.	Allendale, S. C.
N–30	do.	do.	V–58	do.	Virginia

[a] V—refers to variant type in original isolation.
N—refers to normal type in original isolation.

During the course of the experiment, which involved 22 weekly transfers, all of the 27 isolates gave rise to at least 1 variant, while some produced as many as 5 distinct cultural types markedly different from one another and from the original culture. The cultures, as they appear at the end of the experiment, are shown in figure 2, A and B. As the experiment progressed the variants appeared to fall into two rather definite classes, and an additional heterogeneous class. The class containing the greatest number was characterized by a dense white aerial mycelium. The color of the subaerial mycelium and the growth rate of the cultures of this class was somewhat variable. At the completion of the experiment, cultures 4, 6, 7, 11, 12, 23, 24, 32, 39, 40, 45, 48, and 58 could be placed in this general group designated as Class I. The second class was distinguished by a sparse growth of aerial mycelium closely appressed to the surface of the agar. These colonies were

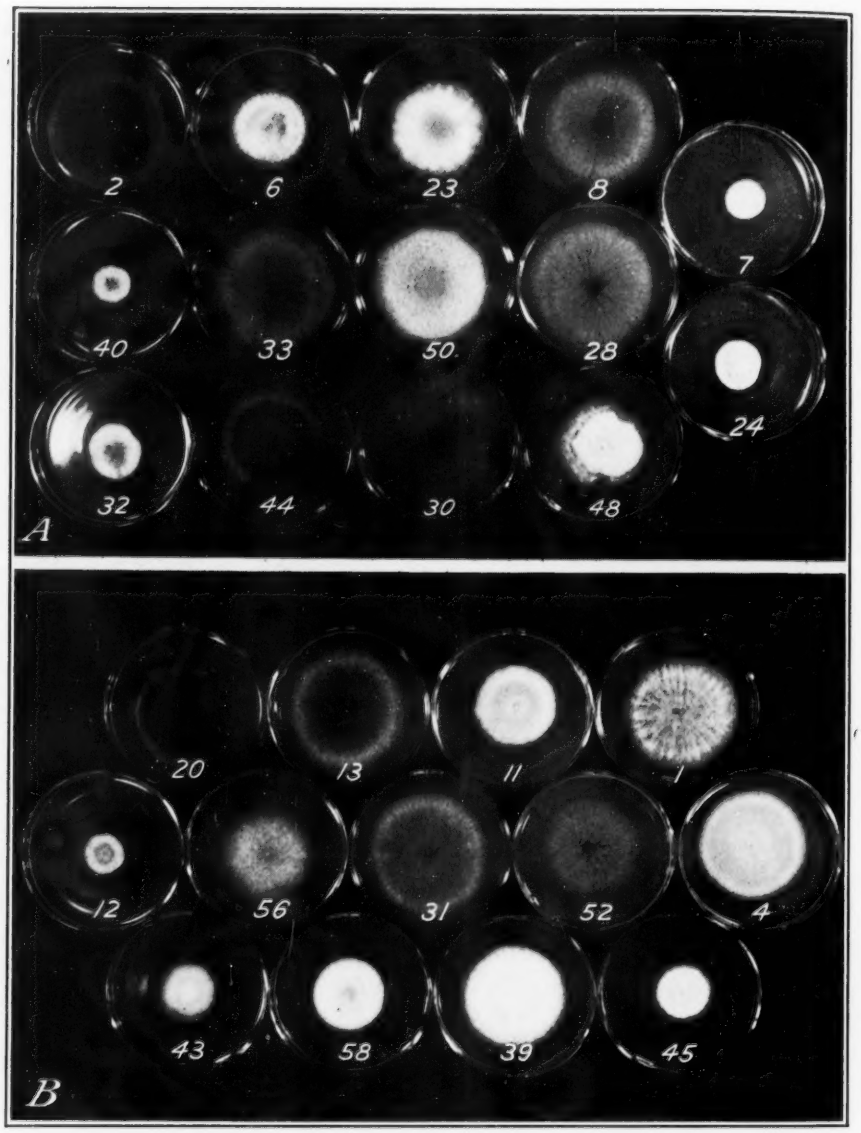

FIG. 2. Variant cultures that arose from the isolates shown in figure 1, after 22 weekly transfers. A. Variants from the "normal type" isolates. B. Variants from the "variant-type" isolates. The colonies are in the same relative positions as the parent isolates shown in figure 1.

buff with irregular, radiating streaks of gray. The cultures that at the end of the experiment could be placed in Class II were Nos. 8, 28, 30, 31, and 52. A third class comprised a heterogeneous group of cultures differing from one another and unlike either those of Class I or Class II. Cultures 1, 2, 13, 20, 33, 43, 44, 50, and 56 were placed in Class III.

When an isolate once changed in its cultural behavior from that found on first isolation, it never reverted to the original type. Variant types of

Class I never were observed to give rise to a variant type possessing the characteristics of Class II. Conversely, variants of Class II never produced variants that could be placed in Class I. Isolates of both these classes produced in turn variants of the heterogeneous type of Class III, and those of the latter class often gave rise to colonies typical of those in Class I or Class II. The variations observed could in no way be correlated with the geographical origin of the isolate nor the particular plant part from which the isolation was made. In several instances variations were in a retrogressive direction; the colonies frequently becoming smaller with each successive saltation. The great majority of variants appeared as entire colonies; few arising as sectors in the parent colony. In 2 instances the conidia of cultures V–13 and V–44 failed to grow when isolated. These cultures could be maintained only by mass transfers of large numbers of spores and mycelial fragments. Eventually, they produced other variants in which the spores grew normally on isolation. Culture V–56 gave rise to a variant completely devoid of conidia and could be propagated only by mass transfers of mycelium or hyphal-tip isolations.

CYTOLOGICAL OBSERVATIONS

Cytological preparations of the conidia were made for the purpose of observing their nuclear condition, and for obtaining some insight as to the nature of the variations under consideration.

A heavy spore suspension was spread over slides that had been smeared with egg albumen. Some of the slides were placed at once in a moist chamber to permit germination of the conidia, others were placed in a fixing solution immediately after the suspension had dried. After fixing in a 6 per cent solution of mercuric chloride containing 2 per cent glacial acetic acid, the spores were stained in Mayer's Haem-alum, dehydrated, and mounted in balsam.

Resting conidia were found to be invariably uninucleate. Although no division figures were observed, the first step in germination appears to be

FIG. 3. Resting and germinating conidia of *Glomerella gossypii* showing the uninucleate condition in each cell. a. Appressorium. Drawings made with the aid of a camera lucida. Objective, oil immersion 95×; Ocular, 6×.

a division of the nucleus followed by the laying down of a wall in the middle of the cell and separating the two daughter nuclei. In germinating conidia each cell of the germ tubes and young hyphae contain a single nucleus (Fig. 3).

PATHOGENICITY STUDIES

The wide variations found in cultural behavior of different isolates, suggested that corresponding differences might well occur in respect to pathogenicity. Experiments, therefore, were undertaken to determine if such variations existed between the original isolates, and whether changes in cultural behavior were accompanied by changes in pathogenicity. It was also of interest to ascertain whether there was any relation between virulence and growth habit.

In a preliminary experiment, 20 of the original cultures and their respective variants were used for inoculation. The original cultures are among those shown in figure 1, A and B. These cultures were maintained at 8°C. from the time of first isolation. The corresponding variant cultures were those that appeared in the final transfer at the completion of the experiment on cultural behavior. These cultures are among those shown in figure 2, A and B.

One variety of cotton, Miller 610, was used throughout the studies on pathogenicity. Preliminary platings and germination tests indicated that this variety was relatively free of internally borne pathogenes, and gave a high percentage of germination. The seed was acid-delinted before using.

Transfers of the original isolates and their respective variants were made to flasks of potato-dextrose agar. When these cultures were incubated 5 days, a spore suspension was made in each flask with equal quantities of distilled water. A suspension of mycelial fragments was used in case of culture V–56, which failed to form spores. In each suspension 100 seeds were immersed an equal length of time and planted with sterile forceps. Controls were set up in which the seed was simply immersed in distilled water. Plantings were made in steamed river sand in flats placed on a bench in the greenhouse. The temperature of the sand was held between 20° and 25°C., and the moisture content maintained at a level that would ordinarily permit normal germination. Variations of pathogenicity, as indicated in greenhouse trials, are shown in figure 4.

Three weeks after planting, seedlings from each inoculation were removed from the sand and classified according to severity of infection. The seedlings were grouped into 4 classes:—(1) healthy, (2) lesioned, (3) post-emergence killing, and (4) pre-emergence killing.

In order to reduce the data to a more simplified form a disease index was used to express the degree of virulence shown by each culture. To obtain a disease index each class of seedlings was assigned an arbitrary value, *e.g.*, healthy = 1, with lesions = 0.5, and dead = 0. The number of seedlings falling in each class was multiplied by the respective class value,

the products added together, and the sum subtracted from 100. Thus a disease index of 100 indicates that all seedlings were killed. Pre-emergence or post-emergence killing was not distinguished by the index.

A second experiment was set up using essentially the same methods, but with fewer cultures to allow for replication. In this trial the seeds were grown in glazed 10 inch pots instead of in flats. Fifty seeds were planted in each pot and each inoculation was replicated 4 times. The pots were placed at random on a bench in the greenhouse. Culture V–56, which

FIG. 4. Examples of differences in virulence between isolates as found in pathogenicity trials. Control plantings in each case are on the extreme right. A. Original isolate, 31S (left), and its respective variant, 31R (center), highly virulent. B. Original isolate, 7S (left), pathogenic; its corresponding variant, 7R (center), mildly pathogenic. C. Both original isolate, 52S (left), and its variant 52R (center), mildly pathogenic.

was not used in the preliminary experiment, was employed in the second trial.

The results obtained in the first experiment are shown in table 2: those of the second experiment, in table 3. A comparison of the corresponding indices in both tables indicates a close agreement between the two experiments. In the second trial, however, a higher degree of virulence was shown by all isolates. This probably was because of a lower soil temperature that prevailed while this experiment was being conducted. Isolate V–52S, the only stock culture to show low virulence, was nevertheless highly infective, as shown by the large number of lesioned seedlings. The variant

TABLE 2.—*Results of first experiment on pathogenicity, showing cultures used, class distribution of seedlings in each inoculation, disease index, and diameter of colony of each isolate used*

Treatment	Living		Dead		Disease index	Diameter of colony in cm.
	Healthy	Lesioned	Post-emergence	Pre-emergence		
Ck	86	8	0	6	10	.....
"	82	12	0	6	12	.....
"	78	10	0	12	18	.....
"	84	11	0	5	11	.....
Cult. No. N- 8S	0	0	4	96	100	8.0
" " 8R	0	0	3	97	100	8.0
" " V-20S	0	0	2	98	100	8.0
" " 20R	0	0	4	96	100	8.0
" " N-28S	0	0	4	96	100	8.0
" " 28R	0	0	4	96	100	8.0
" " N-30S	0	0	13	97	100	8.0
" " 30R	0	0	9	91	100	8.0
" " V-31S	0	0	2	98	100	8.0
" " 31R	0	0	15	85	100	8.0
" " V-52S	0	28	46	26	86	5.0
" " 52R	2	35	41	24	80.5	5.0
" " V- 4S	0	0	0	100	100	6.5
" " 4R	0	0	5	95	100	6.5
" " N- 7S	0	0	5	95	100	8.0
" " 7R	8	39	26	27	72.5	2.5
" " V-12S	0	0	5	95	100	6.0
" " 12R	0	16	15	69	92	5.0
" " N-23S	0	0	3	97	100	8.0
" " 23R	0	0	3	97	100	8.0
" " N-24S	0	0	7	93	100	8.0
" " 24R	20	43	9	28	58.5	2.5
" " V-39S	0	0	2	98	100	7.0
" " 39R	0	0	12	88	100	7.0
" " N-40S	0	0	3	97	100	8.0
" " 40R	0	10	64	26	95.0	3.0
" " V-58S	0	0	3	97	100	7.5
" " 58R	0	0	4	96	100	5.0
" " V- 1S	0	7	58	35	96.5	6.5
" " 1R	1	11	52	36	93.5	7.0
" " N- 2S	0	0	2	98	100	8.0
" " 2R	0	0	13	87	100	6.0
" " V-13S	1	0	11	88	99.0	6.5
" " 13R	0	0	6	94	100	8.0
" " V-33S	0	0	2	98	100	8.0
" " 33R	0	0	2	98	100	8.0
" " V-43S	0	0	8	92	100	6.5
" " 43R	4	52	23	21	70	5.0
" " N-44S	0	0	2	98	100	8.0
" " 44R	0	0	22	78	100	6.5

of this isolate, No. 52R, behaved in essentially the same manner. On further examination of the tables it is found that whenever wide differences in virulence exist between the original isolate and its variant, a corresponding reduction in colony diameter is shown by the latter culture. It seems very possible that growth rate is directly related to pathogenicity but is probably not the only factor involved. All divergencies in growth habit from that of the original culture were not reflected in the relative virulence of the variant. While there was some variation between cultures in respect to

TABLE 3.—*Results of second experiment on pathogenicity*

Treatment	Living		Dead		Disease index	Average disease index	Diameter of colony in cm.
	Healthy	Lesioned	Post-emergence	Pre-emergence			
Ck	35	8	4	3	22		
"	34	9	4	3	23		
"	37	4	3	6	22		
"	40	2	4	4	18		
"	38	4	2	6	20		
"	36	8	3	3	20	Ck.	
"	39	5	4	2	17	Av. = 20	
"	35	11	2	2	19		
Cult. No. N- 7S	0	1	27	22	99		
" " N- 7S	0	0	16	34	100		
" " N- 7S	0	1	19	30	99		
" " N- 7S	0	1	20	29	99	Av. 7S = 99.25	8.0
" " 7R	12	10	8	20	66		
" " 7R	23	8	3	16	46		
" " 7R	27	11	8	4	35		
" " 7R	25	9	5	11	42	Av. 7R = 47.50	2.5
" " V-39S	0	3	17	30	97		
" " V-39S	0	3	15	32	97		
" " V-39S	0	0	15	35	100		
" " V-39S	0	14	12	24	85	Av. 39S = 95.00	7.0
" " 39R	1	6	18	25	92		
" " 39R	3	6	19	22	88		
" " 39R	4	6	19	21	86		
" " 39R	2	21	14	13	76	Av. 39R = 85.50	7.0
" " N-24S	0	0	23	27	100		
" " N-24S	0	0	25	25	100		
" " N-24S	0	0	20	30	100		
" " N-24S	0	0	18	32	100	Av. 24S = 100.00	8.0
" " 24R	36	5	6	3	23		
" " 24R	30	6	9	5	34		
" " 24R	37	7	1	5	19		
" " 24R	30	12	1	7	28	Av. 24R = 26.00	2.5
" " V-56S	0	0	11	39	100		
" " V-56S	0	0	14	36	100		
" " V-56S	0	0	13	37	100		
" " V-56S	0	0	8	42	100	Av. 56S = 100.00	7.5
" " 56R	30	4	11	5	36		
" " 56R	35	6	2	7	24		
" " 56R	32	4	5	9	32		
" " 56R	35	1	6	8	21	Av. 56R = 28.25	5.5
" " N-28S	0	0	16	34	100		
" " N-28S	0	0	7	43	100		
" " N-28S	0	0	9	41	100		
" " N-28S	0	0	11	39	100	Av. 28S = 100.00	8.0
Cult. No. 28R	0	0	14	36	100		
" " 28R	0	0	18	32	100		
" " 28R	0	0	20	30	100		
" " 28R	0	0	11	39	100	Av. 28R = 100.00	8.0
" " N-30S	0	1	6	43	99		
" " N-30S	0	0	15	35	100		
" " N-30S	1	0	14	35	98		
" " N-30S	0	0	6	44	100	Av. 30S = 99.25	8.0
" " 30R	0	0	13	37	100		
" " 30R	0	0	15	35	100		
" " 30R	0	1	14	35	99		
" " 30R	0	0	17	33	100	Av. 30R = 99.90	8.0

TABLE 3.—(*Continued*)

Treatment	Living		Dead		Disease index	Average disease index	Diameter of colony in cm.
	Healthy	Lesioned	Post-emergence	Pre-emergence			
Cult. No. V–31S	0	2	27	21	98		
" " V–31S	0	0	6	43	100		
" " V–31S	0	0	15	35	100		
" " V–31S	1	0	14	35	98	Av. 31S = 99.00	8.0
" " 31R	0	0	25	25	100		
" " 31R	0	0	22	28	100		
" " 31R	0	0	18	32	100		
" " 31R	0	0	14	36	100	Av. 31R = 100.00	8.0
" " V–52S	0	35	12	3	65		
" " V–52S	3	33	10	4	61		
" " V–52S	6	24	12	8	64		
" " V–52S	5	26	13	6	64	Av. 52S = 63.5	5.0
" " 52R	1	38	9	2	60		
" " 52R	0	37	7	6	63		
" " 52R	1	33	11	5	66		
" " 52R	0	29	14	7	71	Av. 52R = 65.0	5.0
" " N–33S	0	0	11	39	100		
" " N–33S	0	0	10	40	100		
" " N–33S	0	0	7	43	100		
" " N–33S	0	0	12	38	100	Av. 33S = 100.00	8.0
" " 33R	0	0	5	45	100		
" " 33R	0	0	6	44	100		
" " 33R	0	0	6	44	100		
" " 33R	0	0	14	36	100	Av. 33R = 100.00	8.0
" " V–43S	0	1	15	34	99		
" " V–43S	0	1	30	19	99		
" " V–43S	0	0	19	31	100		
" " V–43S	0	0	24	26	100	Av. 43S = 99.5	6.5
" " 43R	10	30	5	5	50		
" " 43R	13	24	12	1	50		
" " 43R	18	16	7	9	48		
" " 43R	15	21	5	9	49	Av. 43R = 49.25	5.0

sporulation, no relationship could be found between spore production and pathogenicity.

DISCUSSION

In the studies on variation in cultural behavior it has been pointed out that differences in cultural type appeared on first isolation from diseased host tissues. Although the majority of the original isolates were of the same type, some had divergent growth habits. This suggests that variation in cultural behavior is extant in nature as well as under artificial conditions. The variants found on first isolation were not so widely divergent as those encountered in the subsequent studies.

Studies on pathogenicity of different cultures of *Glomerella gossypii* have shown that all of the original cultures, except V–52S, were highly virulent. A number of the variants that arose from the original cultures proved to be only mildly pathogenic. All variations in cultural characters from that of the original isolate were not accompanied by a reduction in virulence. The only cultural character found to be correlated with pathogenicity was that of growth rate or colony diameter.

Although wide variations have been observed in pathogenicity, it seems improbable that such variations are of great practical importance. Under natural conditions where inoculum is abundant, weakly pathogenic cultures are very likely overrun by more virulent forms.

In certain preliminary experiments not included in the present investigation it has been observed that the variety of the host plant has little or no influence on the expression of the relative virulence of any given culture, although apparent differences in varietal susceptibility were observed. This suggests that selective pathogenicity, as found in several plant pathogenes, does not exist in *Glomerella gossypii*.

The nature of variations as observed in *G. gossypii* appears to be concerned with mutation of the heritable material of the nucleus. The facts that variations occurred suddenly, were irrevertable, and tended to be in a retrogressive direction, suggest that fundamental alterations of the nuclear content are involved. Since the conidia are uninucleate it is obvious that heterocaryosis with subsequent reassortment of nuclei[2] is an untenable explanation of variation in this instance. The character of the observed variants is not that that would be expected if environmental factors were functional in bringing about such changes. It is doubtful whether the perithecial stage of this fungus, through sexual combination and segregation of heritable factors, is involved in the production of variant types in nature. Although the perfect stage has been observed in nature on one occasion,[3] no further reports of its occurrence are to be found. If the perithecia form under field conditions they probably do so only in rare instances.

SUMMARY

Variations in cultural behavior of *Glomerella gossypii* were observed on first isolation from diseased tissues, suggesting that such variant types occur in nature.

Further studies showed that all cultures employed gave rise to at least one variant during the course of the investigation. Of the variants observed the majority could be grouped into 2 rather definite classes on the basis of their growth characters. The largest class was characterized by a dense, white, cottony aerial mycelium. A second class was composed of cultures having sparse buff-color aerial mycelium.

All of the original cultures but one were highly pathogenic on cotton seedlings. Some of the variant cultures were only mildly virulent. Low virulence was directly correlated with slow growth rate of the culture.

Cytological observations revealed the conidia and hyphal cells to be uninucleate. This fact, together with the characteristic type of variation, suggests that the changes in both cultural behavior and pathogenicity are probably due to mutation of the heritable material of the nucleus.

[2] Hansen, H. N. and R. E. Smith. The mechanism of variation in imperfect fungi: Botrytis cinerea. Phytopath. **22**: 953–964. 1932.

[3] Edgerton, C. W. The perfect stage of the cotton anthracnose. Mycologia **1**: 115–120. 1909.

The variations found in cultural characters and virulence are believed to be of little importance from a practical standpoint.

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A STUDY OF FORKING IN RED PINE[1, 2]

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(Accepted for publication July 12, 1938)

INTRODUCTION

A high percentage of false bifurcation of the current season's growth of red pine, *Pinus resinosa* Ait., was noted in a forest planting on the eastern side of Canadice Lake, N. Y., by J. A. Brock in the spring of 1933 (9). The writer found the same disturbance in 3 counties in western New York State, in the northern Adirondacks, and in 2 counties in southeastern Pennsylvania in the summers of 1936 and 1937, although no systematic attempt was made to scout for it. H. H. York has reported it as occurring in plantings in Connecticut and several counties in central and eastern New York.

Unless specifically designed otherwise, all data included in this paper were obtained from plantings on the Rochester Municipal Watershed in Livingston and Ontario Counties, N. Y. Trees in these plantings varied from 5 to 25 years of age.

Kienholz (10) described a type of fasciation that might be considered as a tendency of the species to susceptibility to morphogenic disturbances, but there seem to be no references in the literature to the phenomenon of forking. Witches'-brooms do not appear to have been reported as occurring on red pine, although the writer observed one near the top of a tree in a 22-year-old planting.

SYMPTOMS

The first apparent symptom of forking is the extra-seasonal growth of lateral buds in the winter-bud cluster of the terminal shoot of the tree (Fig. 1, B). Extra-seasonal growth becomes noticeable in late June or

[1] A dissertation presented to the faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] The writer wishes to express appreciation to Prof. H. H. York for advice and criticism during the course of this investigation, and to Prof. J. R. Schramm and Prof. Walter Steckbeck for criticism of the manuscript. Grateful acknowledgement is made to Mr. J. A. Brock for cooperation in the field investigations.

[3] The greater part of this investigation was carried on during the tenure of a George Leib Harrison Fellowship at the University of Pennsylvania.

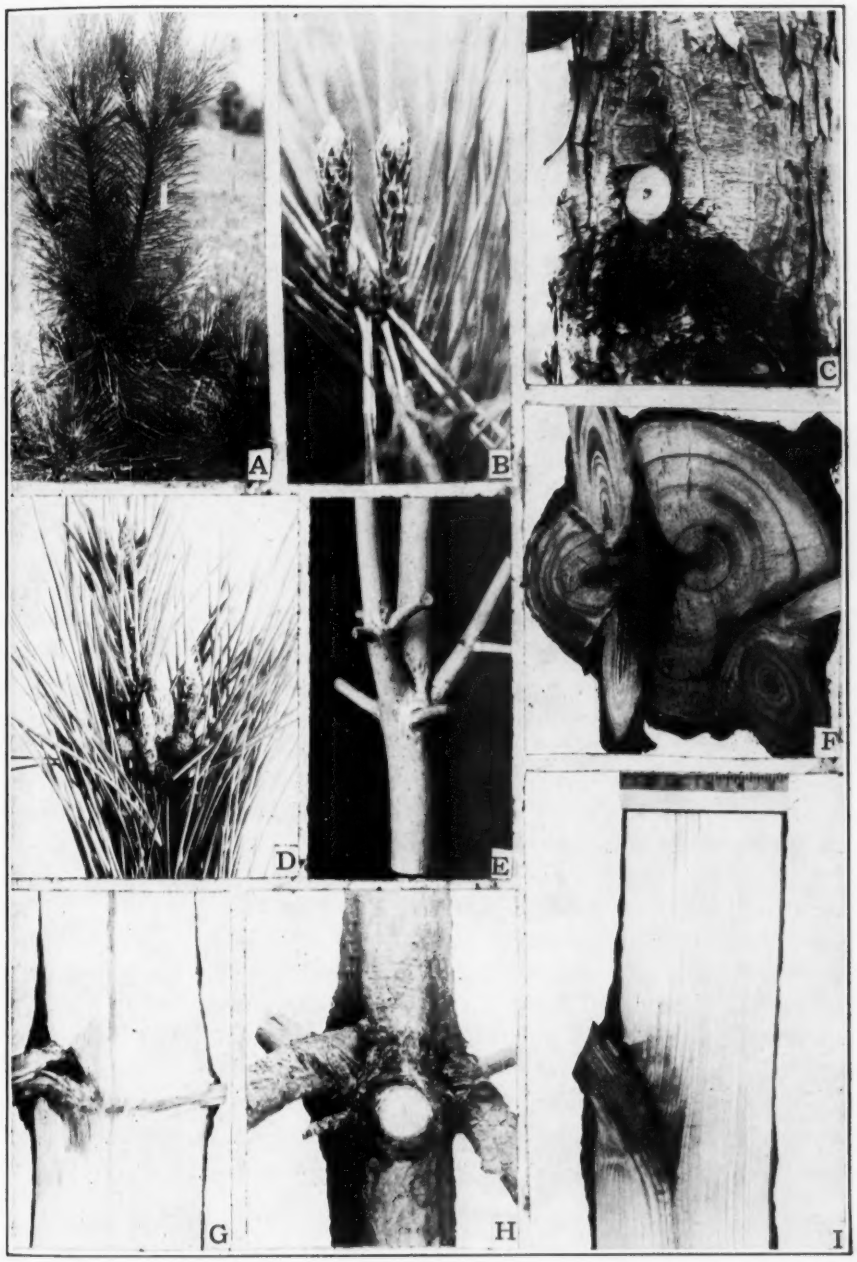

FIG. 1. A. Typical fork in 7-year-old red pine. B. Extraseasonal growth of lateral buds taken July 5 near Springwater, N. Y. C. Pruned forked branch on a 22-year-old tree with depression immediately above. D. Precocious shoot rising from bud cluster with short needles and two buds at apex. E. Peeled fork showing secondary branching. F. Cross section near a fork upon which secondary branching had occurred. G. Radial section of trunk showing effect of ice breakage upon a forked branch. H. Effect of weight of ice upon two forked branches near top of 22-year-old tree. I. Radial section showing characteristic appearance of a fork, with reddish-brown stain extending into the wood.

July, when 1 or 2, and in some cases as many as 4 lateral buds commence to grow. The resulting precocious shoots may attain a length of 3 to 4 inches, with 1 or more winter buds at their apices, before becoming dormant (Fig. 1, D). Needles develop on such shoots, but seldom attain more than half of their normal length and fail to elongate further at the initiation of growth the following spring. The precocious shoots may continue to elongate until quite late in the current season, although, according to Gustafson (5), elongation in pine shoots has been completed by early July in Michigan. Measurements of 10 precocious shoots in a planting of 8-year-old red pine were taken on August 15, 1937, and again on October 25. All but one of them showed an increase in length from $\frac{1}{4}$ to $1\frac{3}{4}$ in. The formation of a bud cluter at the tip of the precocious shoot (Fig. 1, D) interfered with normal subsequent growth, as it caused secondary branches to be formed within a few inches of the bole (Fig. 1, E).

The terminal bud of the cluster in which the extra-seasonal growth occurs seldom takes part in the abnormal development, and usually elongates at the normal time the following season. The laterals, however, during the course of their extra-seasonal growth, react as if the terminal bud had been injured or removed and grow in a more or less vertical direction. Thus the shoot, originating from the terminal bud the following season, grows in competition with one or more laterals that had commenced growth the preceding summer. This results in a bi- or trifurcated appearance of the tree, which will be referred to as "forking" (Fig. 1, A). This condition does not remain conspicuous for more than 2 or 3 seasons in most cases. Either the true leader or one of the precocious laterals will assume dominance and the other member, or members, of the fork will tend to the more or less horizontal position normally characteristic of red pine branches.

Forking may occur repeatedly in a tree. One 22-year-old specimen, when dissected, showed this phenomenon in at least 12 nodes (Fig. 2). An examination of nodes where forking had occurred at some previous time frequently showed a slight depression in the trunk immediately above the insertion of the branch (Fig. 1, C).

There seems to be no relation between the age of a tree and its susceptibility to forking, in stands up to 25 years of age. No data have been obtained upon older trees because of the difficulty of making certain, without felling the tree, that a fork might have been induced by insect or mechanical injury.

DAMAGE RESULTING FROM FORKING

The structural injury that results from forking usually has its inception during the season following the formation of the fork, and is caused by the failure of the forked members to completely unite as they increase in diameter. Because of the acute angle at which they stand in relation to each other, they are unable to slough off, or push aside, the bark that lies on the inside of the fork between their cambiums, in the manner that the growth of the trunk displaces the bark of a normal lateral branch. This

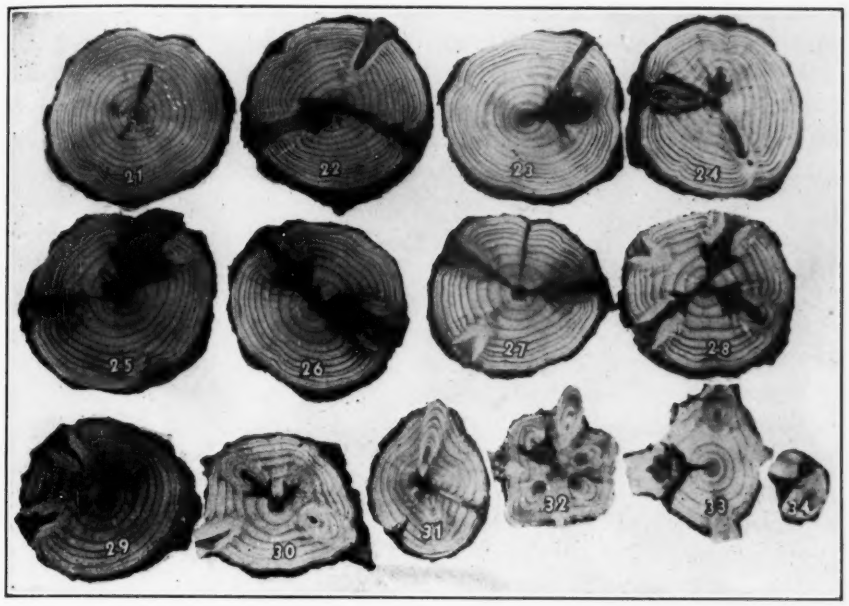

FIG. 2. A series of sections taken from a 23-year-old red pine in consecutive years from 1921 to 1934 inclusive, to show occurrence of forking and discoloration surrounding the pith.

area of non-union of cambium remains in the main axis of the tree as a fissure or pocket that becomes filled with a heterogeneous material composed largely of resin and fragments of the bark (Figs. 1, F and 1, I). These fissures are in a more or less vertical plane for several years until one of the forked members assumes dominance, as described above. While in this vertical position they are easily penetrated by water, and a favorable environment is thus established for the development of many species of parasitic and saprophytic bacteria and fungi. The presence of this flora may be an additional factor in causing the fissure to remain open.

Although it is not within the province of this investigation to evaluate the damage caused by forking from the point of view of the defects in the timber of forked trees, mention should be made of the increase of winter damage in forked stands. Since part of a forked branch may not be united organically with the trunk, the mechanical resistance to stress caused by weight of snow or ice is reduced. A branch that breaks in such a manner (Fig. 1, G) produces a more serious injury to the tree than does the breaking of a sound branch, as it lays open a cavity that may extend almost to the pith (Fig. 1, H). Normal branches are able to stand much greater stress and, if breaking occurs, usually snap off at a point beyond the trunk.

FORKING IN RELATION TO ENVIRONMENT

There appears to be no marked correlation between forking and site conditions, except a possible relationship with the mean seasonal temperature

of the locality. Determinations of soil pH in plots of forking red pine showed a range from 4.5 to 7.5 without appreciable difference in the percentage of forked trees. The disturbance occurred on soils varying from sandy types in parts of the Adirondacks to the clayey loam upon which the pine had been planted in Montgomery County, Pennsylvania. The most prevalent soil types on the Rochester Watershed were Volusia loam and Volusia shale loam. Degree and aspect of slope likewise seemed to be of no significance. There was no relation between forking and extremes of precipitation, or years of abnormal temperatures. The lowest percentages of forking encountered were in natural stands and plantations of red pine near Mt. Discovery, Essex County, New York. This locality was the most northern and the highest in altitude of those studied, which suggested that temperature and latitude might be possible limiting factors of the disturbance. Numerous observations of comparable sites, however, are necessary before any positive statement can be made on this point. Obviously, a shorter growing season would tend to minimize the effects of extra-seasonal growth, even though it might have no effect upon the primary cause.

FUNGI FOUND ASSOCIATED WITH FORKING

Isolations

Cultures were made from red pine wood immediately after sawing. The surface of the wood was sterilized by swabbing with alcohol and flaming an area of several square inches in the vicinity of the place from which the sample was to be taken. A small piece of wood in the desired locality was then excised with a sterile woodcarving gouge, flamed briefly, and thrust into a tube of 2 per cent Fleischman's Diamalt Agar.

Cultures from buds were made by dipping the bud in 70 per cent alcohol and flaming it until the alcohol was burned off and the bud scales were partly charred. The bud was then split longitudinally with a sterile scalpel and the freshly cut surfaces were placed in contact with an agar slant.

Blue-green molds commonly occurred in cultures from discolored wood from the interior of a bole in the vicinity of a fork. The majority of these are believed to be inhabitants of the wood, and not the result of poor laboratory technic, for they usually did not appear for ten days or more following inoculation of the tube of medium with the wood sample. Bacteria were occasionally present, but no attempt was made to examine them in detail, although it was ascertained that no given form was consistently isolated. While only a limited number of trees above 20 years of age could be removed from the plantings for detailed examination, some indication of the internal condition of about 30 forked trees was obtained by means of an increment borer. Frequently, it was impossible to remove a complete core with the borer because of wood rot in the vicinity of old forks about waist high on the tree. Forked areas were almost invariably accompanied by reddish brown streaks within the wood (Figs. 1, I and 2) from which fungi could be isolated. These streaks often were found surrounding the pith in forked trees.

A species of *Tympanis,* closely resembling *T. pinastri,* was cultured in several cases from discolored wood within the trunk adjacent to fissures caused by forking, and also from the interior of forked branches near their origin in the interior of the trunk. In one instance this species was cultured from the wood of a 7-year-old tree that was, apparently, in a perfect state of health, except for a fork that had occurred 2 years earlier.

Comparison of the species of *Tympanis* found in this investigation with cultures of *Tympanis* sp., kindly furnished by Dr. J. R. Hansbrough, indicated that the organism was the same species that was involved in his studies of the Tympanis canker of red pine (7, 8).

There is considerable evidence that *Tympanis* enters the tree, in the majority of cases, through fissures resulting from forking. The writer also believes that the depressed areas on the trunk above the insertion of a branch may, in many instances, result from the internal fissure caused by forking. As such depressions may, according to Hansbrough, be a symptom of *Tympanis,* a careful study of the internal anatomy, in addition to cultural investigations, seems advisable before making a diagnosis in the absence of fungus-fruiting bodies. Depressions accompanied forked branches in the majority of cases studied, in which the node was 10 or more years of age. Observations based upon the external appearance of depressions of 118 trees showed that the branch at the base of the depression had been a member of a fork in at least 84 per cent of the cases. The true figure probably would approach 100 per cent, since previous forking could not always be determined from external observations on account of the size and age of the nodes near the base of large trees and close pruning of lower branches in the stand.

Association of Dematium pullulans with Forking

The organism most consistently cultured from forked red pine was identified by Dr. David Linder of the Farlow Herbarium as a variety of *Dematium pullulans* de Bary.

Dematium pullulans was cultured from the lateral and terminal buds, pith, discolored wood surrounding the pith, and discolored wood in the root crown of red pine from the Rochester Watershed. It was also cultured from buds of forked red pine growing in southeastern Pennsylvania. It was the only organism to develop in significant percentages from red pine buds. Approximately 75 buds were used as material for culture, and 64 per cent of the resulting organisms proved to be *D. pullulans.* It also was found occasionally in cultures from other parts of the tree: twice in forked branch traces, twice in the pith, and once in the root crown. Cultures from the roots failed to show *D. pullulans,* although it is possible that it was not noticed because of the vigorous growth of bacteria and blue-green molds in some of the cultures. Although positive identification of the fungus in the tissues could not be made by means of an histological study, the presence of a catenate type of hyphae in the pith (Fig. 4, F) strongly indicated that the fungus under observation was *D. pullulans.* The probability of the

accuracy of this determination was strengthened by the lack of any cultural evidence of the presence of fungi possessing a mycelium of this type other than *D. pullulans*. Resistance of the hyphal wall to Cartwright's stain (1), which characterizes the chromatic hyphae of *D. pullulans*, was also typical of the pith-inhabiting organism.

Several members of the *Dematiaceae* have been recognized for some time as wood-inhabiting organisms. Siggers (13) refers to *Torula ligniperda* as a cause of structural weakness and brown staining in tulip poplar and white ash. Reddish-brown staining was usually present in the vicinity of old forks in red pine wood and was of frequent occurrence surrounding the pith in trees affected by the disturbance (Fig. 2).

Dematium pullulans is readily grown upon a wide variety of culture media, both liquid and solid. Cultures in flasks on sterile red pine sawdust, moistened with distilled water, produced a mycelium and conidia, but the yeasty conidial masses, characteristic of its growth on other media, were not evident. A series of cultures was made by placing a block of agar 5 mm. square, containing the fungus, upon the surface of steam-sterilized soil in large culture tubes. Growth took place readily and the hyphae penetrated the soil to the bottom of the tube: a depth of about 6 cm. Single-spore cultures were made by means of the Kienholz technic (11), and the following observations were made on sub-cultures of these on prune agar and on liquid 2 per cent malt.

MORPHOLOGICAL OBSERVATIONS UPON DEMATIUM PULLULANS

Dematium pullulans de Bary: The mature conidia are typically ovoid to ellipsoidal, averaging $7.4 \times 4.0\,\mu$, 2-guttulate and nearly hyaline. Variations from this type occasionally occur (Fig. 4, A), especially in liquid culture. In hanging-drop cultures the spores may reproduce by budding or germinate directly, forming hyphae upon which conidia may be borne laterally (Fig. 4, E). Freshly transferred cultures on prune agar produce conidia so abundantly that a yeasty mass, varying from white to light violet, is formed upon the surface of the culture. The vegetative mycelium is usually somewhat submerged and black in macroscopic appearance, forming a compact stroma-like layer at the surface. The mycelium varies from a dark *Torula*-like form, frequently found in liquid cultures, to a hyaline, distantly-septate type. It may contain granular material or drops of oil in some instances (Fig. 4, E). Round, black bodies, usually less than 1 mm. in diameter, often appear on the surface of prune-agar cultures 2 weeks or more old. These structures are composed of a black, highly-compacted outer layer with a pseudo-parenchymatous, readily stainable central core. Such bodies will be referred to as microsclerotia. Dark-ringed tapering hyphae often appear in tufts on the surface of microsclerotia, although they may also arise directly from the mycelial surface. Bulbilous structures, apparently of similar nature to the rings, frequently occur at the base of a hyphal branch. A type of exospore, resembling spores of the genus *Coniothecium*,

was found among these hyphae and was abundant also in liquid cultures. These spores were ovate to orbicular and usually very dark. They averaged 24 μ in length, and often had one or more hyphal cells attached to their distal end.

A graded pH series was set up with Elliott's medium (3), which contains asparagin as a nitrogen source; and a second series was run with 2 per cent malt. The agar was omitted from these media and the pH was adjusted with HCl and NaOH. The malt series of 12 pH values from 1.8 to 8.5 showed growth from 1.8 to 7.2, although growth was slow at the extremes of the range, particularly at the acid end. Optimum growth occurred between 5.5 and 6.5. On Elliott's medium at pH values from 2.2 to 8.4, growth was obtained from 2.2 to 7.2, with an optimum range betweeen 5.0 and 6.5. The malt series was inoculated with mycelium, while conidia were used with Elliott's medium.

Growth of different cultures of the fungus collected from various localities showed no tendency toward morphological variation when grown upon 2 per cent malt agar in Petri dishes. Numerous varieties of *Dematium pullulans* have been described, but the variety with which we are here concerned cannot be placed satisfactorily among these. In the character of its nodose hyphae it resembles var. *fimicola* Marchal, but differs markedly from it in spore characters.

EXTENT OF FORKING IN STANDS

A partial summary of data obtained from some of the plots studied on the Rochester Watershed is given in table 1. It is evident from data upon plots C and D that the more vigorous trees are the most susceptible to forking. This seemed to apply to trees in all of the age groups studied, although quantitative data are not available for trees of the older classes.

TABLE 1.—*Amount of forking with related data on sample plots*[a] *on the Rochester watershed*[b]

Plot	Trees examined	Age	Trees forked	Trees with precocious buds in terminal 1937 cluster	Trees with secondary lateral near main stem	Average height of stand	Average height of forked trees
	No.	*Years*	*Per cent*	*Per cent*	*Per cent*	*In.*	*In.*
A	417	10–11	92	.....	.....	121	.....
B	100	7–8	63	.....	.....	.....	.....
C	404	6–8	69	.....	.....	27.1	30.0
D	100	5–7	78	30	40	33.1	34.5
E	60	5–7	88	10	38	37.1	37.1
F	88	6–10	92	30	51	.....	.....
G	100	23–25	94	.....	22	324	.....
H	100	7–9	87	41	57	.....	.....

[a] All plots represent different plantings except E and F which are from the same lot.
[b] Blanks in the table indicate that no data were taken.

A map was made of two plots (C and G, Table 1) to determine whether foci of infection occurred in forked stands. The map indicated that forking was evenly distributed throughout the stand, as no evidence of "islands" was found. Two stands of natural red pine within 30 miles of the watershed showed percentages of forking that fell in the range of those of the plantings. In most cases the plantings were of pure red pine, although in some of them there was a light admixture of Norway spruce and eastern white pine, which apparently had no effect upon the prevalence of forking.

The disturbance seems to reach a maximum after a period of years and then gradually subside, although there are indications that there may be a second cycle in the same planting. Annual occurrence of forking when plotted on coordinates (Fig. 3) may be considered additional evidence that an organism was the causal agent of the disturbance.

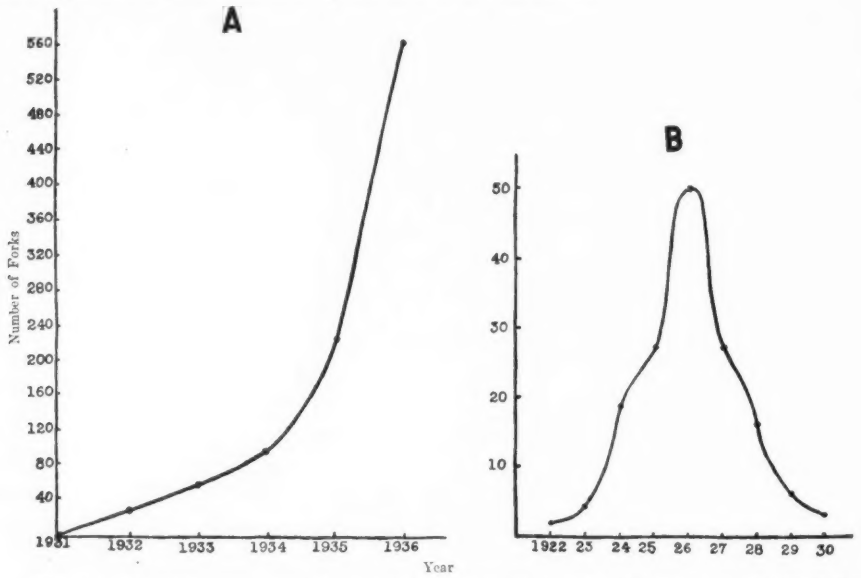

FIG. 3. Annual occurrence of new forks in plantings of red pine. A. Distribution of forks on 852 trees, 6–7 years old, from five plantations. B. Distribution of forks on 100 trees from a plantation in which the trees averaged 23 years old.

HISTOLOGICAL STUDIES

Material for histological examination was fixed in a chromic acid—formalin—salcylic acid solution (2), dehydrated with the N-butyl alcohol technic, and cut on a sliding microtome in sections varying from 5 to 15 μ in thickness. It was found necessary in working with the older tissues to extend the period of dehydration and infiltration to 10 days or 2 weeks in order to insure complete penetration by the alcohol and paraffin. Buds, precocious shoots, wood from the older portions of the trunk, and roots were examined for abnormal anatomical features and for the presence of fungus hyphae. The most successful stain employed was Cartwright's picro-analine blue combination (1), although the chromatic hyphae that appeared in the old wood

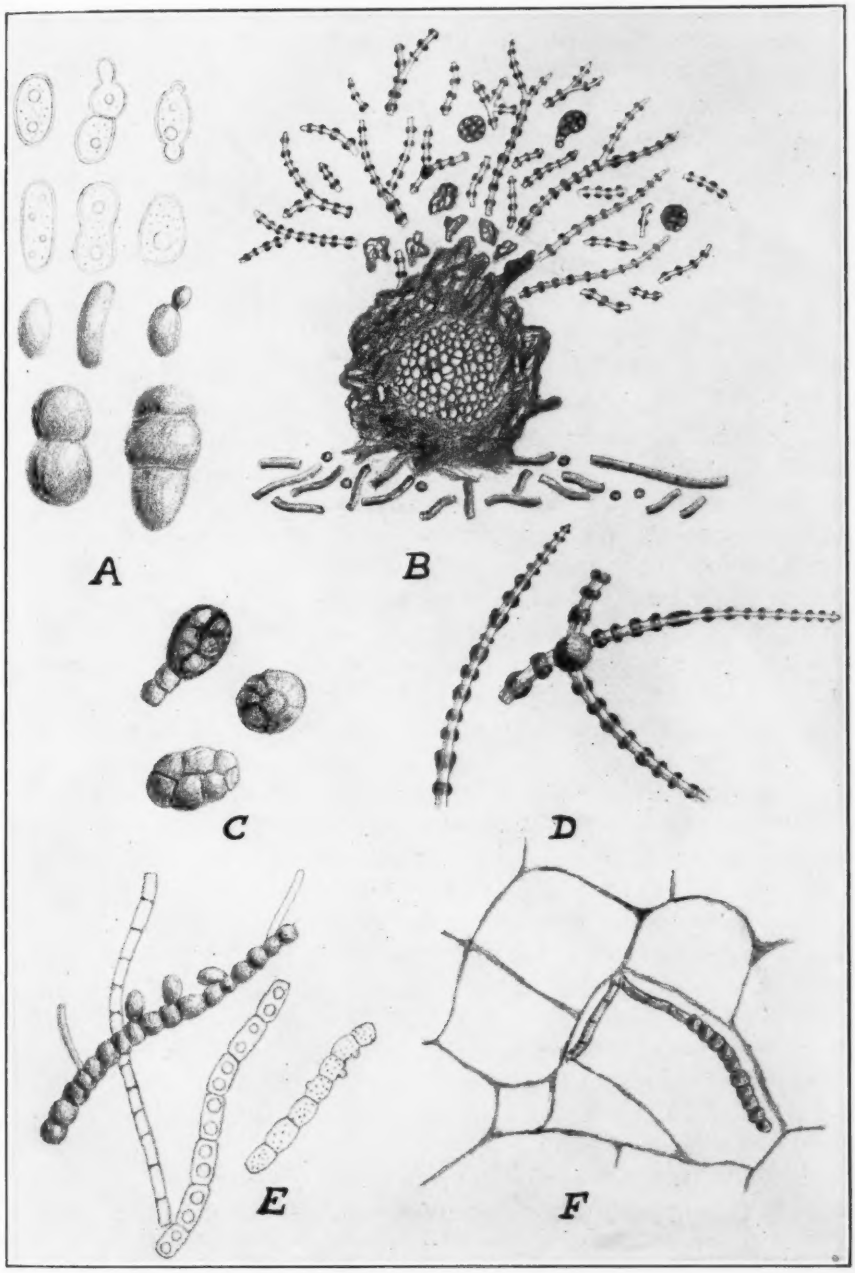

FIG. 4. *Dematium pullulans* de Bary. A. Types of conidia; the spore in the upper left is the typical form. B. Section through a microsclerotium with nodose hyphae and *Coniothecium*-like spores. C. *Coniothecium*-like spores as found among nodose hyphae and in liquid culture. D. Nodose hyphae. E. Types of hyphae. F. Hyphae, believed to be of *D. pullulans* in pith cells of *Pinus resinosa* (drawing from camera lucida).

were satisfactorily demonstrated in unstained sections. No hyphae were observed in the root sections, but chromatic *Torula*-like hyphae were abundant in the pith of the wood taken near a twelve-year-old node (Fig. 4, F) and occasionally penetrated the wood rays. Hyphae occurred both inter- and intra-cellularly in the pith, and were observed entering pith cells through the simple pits in the walls. Minute hyphae also occurred in the procambium and primary xylem of precocious buds, but were not found in the undifferentiated tissue of the promeristem toward the apex of the bud. Such hyphae appeared to be entirely intercellular. There was no evidence of destruction of cells or of the occlusion of vessels in any of the infected material.

INOCULATIONS

A suspension of conidia of *Dematium pullulans* in distilled water was used to inoculate a series of potted red pines 5 years of age. These trees were brought into the greenhouse from coldframes in early January and inoculated about a week later. Seasonal growth was initiated in the trees, in most cases, by the second week of February. The conidial suspension was introduced into the bud, and into the phloem and pith of the stem by means of a hypodermic needle. Distilled water alone was injected into 3 trees of the series to serve as a control. The site of the needle puncture was ringed with a colored wax pencil, and 5 weeks after inoculation the buds and sections of the stem were surface-sterilized, split longitudinally, and placed in tubes of nutrient agar. Sections of the stem were taken from the site of the inoculation and at points 1 and 2 cm. above and below it. Each tree bore 3 inoculations: in the terminal bud; 1 to 2 in. below the bud; and 8 to 14 in. below the terminal. *D. pullulans* was recovered from all of these sites and, in some cases, from sections 1 and 2 cm. above and below the inoculation.

Another series of potted pines was inoculated by the following method. A small area of the stem was surface-sterilized with 70 per cent alcohol and a slight wound was made in the bark with a sterile needle. This wound penetrated the cells of the cortical parenchyma but did not reach the cambium. A small piece of agar upon which *Dematium pullulans* was growing was placed in contact with the wound; absorbent cotton, wet with distilled water, was placed over it; and a band of cellophane was wrapped around the cotton and tied at both ends to insure a moist atmosphere around the inoculum. The cotton and cellophane were removed after 2 weeks, and five weeks from the date of inoculation an attempt was made to recover *D. pullulans* from the stem in the manner previously described. The failure of *D. pullulans* to develop in any of these cultures leads to the conclusion that it is unable to enter the tree through wounds in the bark or cortical parenchyma. Since resin canals are abundant in the latter tissue, it is possible that the copious resinous exudation may have a fungicidal action.

DEMONSTRATION OF A GROWTH SUBSTANCE PRODUCED BY DEMATIUM PULLULANS

Dematium pullulans when grown in liquid culture produces abundant

growth substance, according to the lupine test developed by Granick and Dunham (4). This technic briefly consists of measuring increase of growth in length of the upper centimeter of a 7–8 cm. etiolated lupine hypocotyl, decapitated at the junction of the hypocotyl and the cotyledons. The substance to be tested is applied to the cut surface in lanolin paste or agar and a mark is made with India ink 1 cm. below the cut. The increase in length of the original centimeter is measured after 4 days exposure to light. For this study *D. pullulans*, *Tympanis* sp., *Fusarium* sp. and *Polyporus schweinitzii* were grown on liquid 2 per cent malt in 500 cc. Erlenmeyer flasks until a thick hyphal mat covered the surface of the medium. The mat was then removed, washed in water to free it from as much of the malt as possible, and dried thoroughly at a temperature not exceeding 80° C. Fleischman's yeast was grown on Pasteur's solution instead of malt, and freed of the medium by washing in a funnel lined with filter paper. The dried mats were ground in a mortar, and the resulting powder was mixed in a proportion of 2 parts by weight of lanolin to 1 part of the fungus powder. The increase of growth produced by the powder of *Dematium pullulans* was compared with that of other fungi and with control plants upon which lanolin alone was applied. Several series of tests were conducted at intervals of 2 weeks and it was found that the average increase of growth produced by a given organism varied slightly in the different series, but the relative amounts of growth produced by the fungi employed remained constant. This variation might well have been caused by increasing greenhouse temperature and change of season during the experimental period. Fifteen to 30 test plants were used for each species of fungus tested. *Dematium pullulans* produced a greater increase of hypocotyl elongation than any of the other fungi tested. The relative growth-promoting activity of the powdered mats of the fungi may be expressed as follows: *Dematium pullulans* > Fleischman's yeast > *Fusarium* sp. > *Tympanis* sp. > *Polyporus schweinitzii* > controls.

DISCUSSION

The causal agent of forking cannot definitely be determined from the experimental data available at present, but the theory of a fungus origin seems to be the most reasonable. Possible insect injury, genetic factors, or physiological disturbances of a nutritional nature do not appear to provide a satisfactory explanation for the phenomenon.

Complete serial sections of buds were examined, and no traces of larvae or the puncture of insect proboscl could be found. There was no evidence of insects on the buds in the course of daily field observations made during late June and the month of July. Even if this evidence were disregarded, an hypothesis of insect injury would have to imply a selective feeding of the organism, since the terminal bud does not ordinarily show extra-seasonal growth. Such a circumstance seems highly improbable.

A physiological disturbance from the standpoint of a nutritional unbalance or excess cannot be correlated satisfactorily with the wide range of soil

types of varying hydrogen-ion concentrations upon which forking occurs. Gustafson (6) found that red pine, brought into the greenhouse in January, would commence growth at once and form 2 bud clusters by July. Apparently the terminal bud develops normally in this case, and there would seem to be no connection with the type of growth that results in forking.

It cannot be proved conclusively that a genetic factor might not be causally connected with forking, but several circumstances are contrary to such a possibility. The wide geographic range of the occurrence of forking, together with the fact that forking in neighboring stands may gradually attain a maximum in different years and at different ages of the plantings (Fig. 3), indicates genetic influence to be unlikely.

The presence of fungi in the buds and internodal pith, as indicated by the results of cultural and histological studies, forms the basis of the writer's suggestion of a theory of the fungus origin of forking. Research on phytohormones has proved that bud inhibition and activation are regulated, at least in some instances, by auxin. It also is well established that certain fungi produce auxin in considerable quantities. Thus, the extra-seasonal growth of laterals might be attributed to the production of auxin within the bud by fungus hyphae. Le Fanu (12) showed that in two shoots of *Pisum*, one of which was being strongly inhibited by the other, the dominant shoot indicated a high concentration of auxin with the *Avena* test, while the inhibited shoot showed only slight traces. If a similar condition occurs in the bud clusters of pine, it would follow that the presence of a phytohormone produced by a fungus in the lateral buds might conceivably act as a stimulus to extra-seasonal growth. Production of the hormone in the terminal bud would not necessarily lead to extra-seasonal stimulation if the hormone were normally present in considerable quantities. The abundant production of growth substance by *Dematium pullulans* and the proof of its ability to inhabit red pine might be regarded as indicative of the possibility that the fungus could be causally connected with the disturbance. It is impossible to compare the microflora of normal trees with those of this investigation. Since forking may be latent in a tree, it could not be determined with certainty that a given specimen was completely normal.

SUMMARY

An abnormal bifurcation of red pine trees is prevalent in New York State and other sections of the northeastern United States. This condition is caused by extra-seasonal shoot elongation in which the lateral buds take part while the terminal remains in the dormant condition. Failure of the inner surfaces of the resulting fork to unite completely during subsequent growth of the tree causes an open fissure in which wood-destroying fungi may enter. *Tympanis* sp. also frequently gains entrance in this manner. No correlation was found between rate of forking and site conditions. Trees at high altitudes in the northern part of the area studied showed the lowest percentage of forking. *Dematium pullulans* de Bary was isolated from

abnormal buds in the majority of cases, and hyphae of the characteristic form of the organism were found in the wood of abnormal trees. Young red pines were inoculated hypodermically with conidial suspensions of *D. pullulans* and, after 5 weeks, the fungus was reisolated from sections of the stem 1 and 2 cm. from the site of inoculation. *D. pullulans* was found to contain a growth promoting substance by means of the lupine technic in a quantity exceeding that of other fungi tested. The theory is advanced that abnormal growth of a pine bud may be initiated through the action of a phytohormone produced by the fungus living within the tissues.

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LITERATURE CITED

1. CARTWRIGHT, K. ST. G. A satisfactory method of staining fungal mycelium in wood sections. Ann. Bot. **43**: 412–413. 1929.
2. COHEN, I. and K. D. DOAK. The fixing and staining of *Liriodendron tulipifera* root tips and their mycorrhizal fungus. Stain Techn. **10**: 25–32. 1935.
3. ELLIOTT, J. A. Taxonomic characters of the genera *Alternaria* and *Macrosporium*. Amer. Jour. Bot. **4**: 439–476. 1917.
4. GRANICK, S. and DUNHAM, H. W. Growth substance determinations. Science (n.s) **87**: 47. 1938.
5. GUSTAFSON, F. G. When does a pine tree complete its seasonal growth? Papers Mich. Acad. Sci. **22**: (1936) 83–84. 1937.
6. ———. The influence of length of photoperiod upon the initiation of growth in *Pinus resinosa* seedlings. (Abstract) Amer. Jour. Bot. **24**: 738. 1937.
7. HANSBROUGH, J. R. A new canker disease of red pine caused by *Tympanis pinastri*. Science (n.s.) **81**: 408. 1935.
8. ———. The tympanis canker of red pine. Yale University School of Forestry Bull. 43. 58 pp. 1936.
9. JUMP, J. A. A new disturbance of red pine. Science (n.s) **87**: 138–139. 1938.
10. KIENHOLZ, R. Fasciation in red pine. Bot. Gaz. **94**: 404–410. 1932.
11. ———. Isolating single spores without special equipment. Phytopath. **27**: 950–951. 1937.
12. LE FANU, B. Auxin and correlative inhibition. New Phytol. **35**: 205–220. 1936.
13. SIGGERS, P. V. *Torula ligniperda* (Willk.) Sacc., a hyphomycete occurring in wood tissue. Phytopath. **12**: 369–374. 1922.

CORN-SEEDLING VIRESCENCE CAUSED BY ASPERGILLUS FLAVUS AND A. TAMARII

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(Accepted for publication June 14, 1938)

A color reaction of corn seedlings known as virescence, heretofore unreservedly attributed to genetic factors, has been fully reproduced by the writers by fungus action on corn kernels that do not carry the *vv* factors. Apparently, the products of metabolism of these fungi or their interactions with the host plant affect the seedlings in an abnormal way in exactly the same manner as do certain genes and may serve as a clue to the manner in which certain genes operate.

After considerable experimentation and numerous successes and failures at reproducing this disease, the writers now feel that the governing factors

are sufficiently understood, empirically, so that the facts warrant publication. Much of the fundamental nature of this disease, which may be called "induced virescence," remains to be learned.

SYMPTOMS OF INDUCED VIRESCENCE

Strongly virescent-reacting plants, resulting from inoculation with the causal organisms, can be detected when the coleoptile projects only 2 cm. from the ground and before the first leaf has emerged. In the 3-leaf stage (Figs. 1, 2) the oldest (lowest) leaf frequently showed the most greening,

FIG. 1. Corn-seedling virescence induced by *Aspergillus flavus*. A. Normal seedling. B. A common type of virescence, greening starting at the tips of the leaves and in the coleoptile. C, D. Green color developing as stripes along the principal veins.

but this was not always true. The extent of chlorophyll deficiency may range all the way from only traces of green in the leaf tips through intermediate stages up to wholly green plants. The areas lacking chlorophyll vary in color from almost white to cream or pale green.

Greening proceeds especially from the tip downward, but it frequently also develops at the same time from the base upwards. The greening may proceed gradually without streaking or it may appear definitely as streaks along the principal veins (Fig. 1). The greening from the base, when it occurs, often proceeds especially along and outward from the midrib. If chlorophyll is very deficient, the plants may die in the 3-leaf stage. If, on the other hand, there is sufficient chlorophyll for continued growth, the roots at the crown develop, and all succeeding leaves after the third or fourth are fully green, although growth in size may, for a while, almost cease (Fig. 3).

Only rarely, and under the most favorable conditions for the expression of virescence, did all the plants in a pot planted with inoculated open-pollinated seed exhibit chlorophyll deficiency. Usually, the reaction was

as is shown in figure 2, where some plants are almost white, some partly green, and a considerable number of them normally green. Temperature, light, soil conditions, extent of seed-coat injury, and concentration of spore suspension, all, had an effect on the extent to which virescence was expressed.

Final notes in greenhouse experiments were made when most of the plants were in the 3-leaf stage. Some greening had taken place by that time, but not enough to obliterate symptoms that showed earlier; and all weak plants that were able to emerge had done so by that time.

FIG. 2. A population of a variety of open-pollinated dent corn, some of the seedlings being much more resistant to "induced virescence" than others. Similar segregation for resistance and susceptibility occurred in all open-pollinated varieties tested.

Plantings made in field plots showed the same kind of variation shown in figure 2. Plants that were half or more green by the time they reached the 3-leaf stage survived for the most part. Some of these were extremely weak (Fig. 3), while others produced more or less grain at maturity.

GENETIC VIRESCENCE COMPARED WITH INDUCED VIRESCENCE

In the course of corn-breeding studies at the Illinois Station, many inbred ears are tested on greenhouse benches. In the seedlings thus produced the writers believe they have seen genetic virescence of every type of appearance coinciding with the symptoms of virescence induced by *Aspergillus*. Twenty types of genetic virescence have been numbered by various investigators (1, 2, 3, 4, 9, 10) and a few additional ones have been studied. Although some of these differ considerably in appearance, many are phenotypically

FIG. 3. A. Young corn plants, both grown from kernels from which the seed coat had been removed from the crown before inoculation with *Aspergillus flavus*. One plant failed to become virescent, while the other developed only enough green to barely survive through the seedling stage, and was in the 6-leaf stage and fully green when photographed. B. The same 2 plants at maturity photographed from approximately twice the distance. The weak plant produced a small tassel and ear shoot but no grain.

either similar or alike and can be distinguished only by their linkage relationships. Only in one case, V_{17}, was greening described as being more pronounced at the base of the leaf than at the tip. In all other cases, where mention of the position is made, greening is mentioned only as beginning at the tip. Lindstrom (9, Plate 1), however, illustrates slight green color at both the tips and bases of nearly white leaves. In none of the brief descriptions of genetic virescence is green striping along the veins, as shown in figure 1, mentioned. This occurred frequently in induced virescence and was also observed by the writers in some types of genetic virescence.

Most writers describing genetic virescence observed that the chlorophyll-deficient plants were smaller than the normal ones in the seedling stage. In measurements of 4460 plants, those exhibiting induced virescence average 1.9 cm. shorter than those similarly treated, but green in color (Table 1).

TABLE 1.—*Influence of seed-coat injury and seed inoculation with spores of Aspergillus flavus on plant height, blighted plants, visible rots, and internal infection in mesocotyl of corn seedlings. Data taken when most plants were in the 3-leaf stage*

Plant classification	Height of plants	Plants blighted whole or in part	Mesocotyl infection		
			Visibly rotted	Total internal infection[a]	Internal infection with *A. flavus*
	Cm.	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Noninoculated control plants					
Seed coat sound	18.7	0.2	5.3	18.8	0.6
Seed coat removed at crown	11.8	0.7	9.7	37.5	0.6
Seed inoculated with *A. flavus*					
Seed coat sound	18.4	0.7	4.2	31.7	18.8
Seed coat removed at crown, plants green	11.3	1.3	18.0	50.0	31.3
Seed coat removed at crown, plants virescent	9.4	5.2	19.5	58.2	48.6

[a] Total infection with various organisms—Penicillia, Fusaria, and Mucors, being prominent in addition to *A. flavus*. Bacteria were largely eliminated by using acidified medium.

In working with "yellowish virescent," Carver (1) found that under field conditions about one-third of the plants matured. These were about half as tall as normal plants and produced some pollen but no ears. This compares very well with the condition illustrated in figure 3. Mature plants from some other types of genetically virescent seedlings produced small and inferior ears (10).

FACTORS INFLUENCING VIRESCENT REACTION

SEED-COAT INJURY

A break in or removal of part of the seed coat of corn kernels before inoculating with spores of *Aspergillus flavus* or *A. tamarii* and planting is a prerequisite to readily incident virescence. Even without artificially injuring the seed coats, if other conditions are favorable, an appreciable number of virescent seedlings usually develop from inoculated seed of corn types with rough indentation, because of seed coat breakage at the crown from normal handling.

Experiments were conducted with various kinds of artificially made seed-coat injuries differing in location and extensiveness. The appearance of these injured kernels was illustrated in another publication (6, Fig. 10). Certain types of seed-coat injury, without seed inoculation, but incident upon simply planting the grain in corn-belt soils, resulted in considerable pathological disturbance to the plants grown therefrom (8).

When the entire seed coat was removed from the crown and the kernels were then inoculated, 69.7 per cent produced virescent plants. Removal of one-fourth the seed coat from the crown caused 44.5 per cent, and a cut of about 1/16 inch across the crown without removing any seed coat caused 20.0 per cent. A cut through the seed coat into the side of kernel and slightly into the horny endosperm, but not exposing soft endosperm, caused 43.5 per cent virescent plants. Breaking off the tip cap from the kernels caused 14.1 per cent, and there were 1.2 per cent virescent plants in the checks with apparently sound seed coats, which also had been inoculated. These are averages from several tests conducted in the winter in a greenhouse and involved approximately 500 crown-injured kernels and the same number of uninjured checks. The station strain of Reid Yellow Dent open-pollinated corn was used in this and all other tests herein reported, except where other types are specifically mentioned.

CORN VARIETIES, INBREDS, AND HYBRIDS

Of 20 varieties and hybrids tested, all produced induced virescence prominently. These included 7 hybrids of dent corn, 4 open-pollinated varieties of dent corn, 3 of flint corn, 3 of sweet corn, 2 of popcorn, and 1 of flour corn. One hybrid was outstanding in producing 100 per cent virescent plants in several repeated tests. The flour corn and the closely selected dent variety known as "Illinois high protein" averaged about 90 per cent virescent plants in the same tests. All of the remainder were somewhat lower but none of them outstandingly low. Only in certain inbreds (5, Fig. 19) was a high degree of resistance to induced virescence found.

LIGHT

That light has an influence on the virescent reaction was demonstrated in 2 tests. Ten 1-gallon jars were each planted with 20 kernels of crown-injured seed inoculated with *Aspergillus flavus*, 10 were inoculated with *A. tamarii*, and an additional 5 were planted with sound noninoculated seed to check on the possibility of the seed carrying factors for genetic virescence. These were placed intermingled on a greenhouse bench, where they received daylight only. A duplicate set received continuous light. When the sun was not shining, 500-watt Mazda bulbs in reflectors placed 3 feet above the plants were used. The greenhouse temperature was 20–22° C.

The first test was conducted during January. Under daylight only, *Aspergillus flavus* caused 50.6 per cent virescent plants and *A. tamarii* caused 38.4. Under continuous light the percentages were respectively 25.8 and 15.5. The second test was made during March. This time, under daylight only, *A. flavus* caused 18.4 per cent virescent plants, while *A. tamarii* caused 16.4. Under continuous light the respective percentages were 10.8 and 6.2. The checks in all cases remained almost free from virescence.

The second test was grown under longer daylight than the first and also produced considerably fewer virescent plants. That the reduction in vires-

cence in the second test, as compared with the first, was due to difference in light alone cannot be stated for certain. There appears to be no doubt, however, that within each individual test conducted at the same time the differences in virescence were caused by differences in amount of light.

TEMPERATURE

Temperature also, is of importance in influencing the degree of virescent expression following inoculation. Experiments in which Wisconsin-type soil-temperature tanks were used were conducted in the spring of 2 different years. In 1936, *Aspergillus flavus* inoculations at a soil temperature of 16° C. produced 56.6 per cent virescent plants. At a soil temperature of 30° C., only 3.2 per cent occurred. In 1937 similar inoculations at 18° C. soil temperature produced 28.6 per cent virescent plants, while, at 30° C. soil temperature, only 1.3 per cent occurred. In all cases the air temperature varied within about 2° of the soil temperatures. Inoculations with *A. tamarii* also were similarly affected by temperature, but not to so marked an extent.

SOIL MOISTURE

Soil moisture is another important factor influencing the development of induced virescence. Experiments were conducted in the greenhouse with different batches of dark silt-loam soil in the early spring of 3 different years. The results were very similar, and only a summary need be given. The growth of corn in dry soil with only about 18 per cent moisture, dry basis, was compared with wet soil of about 27 per cent moisture. The latter soil could still be screened and handled satisfactorily without puddling, and emergence of corn seedlings was 3 to 4 days earlier than in the drier soil.

Inoculation of crown-injured corn kernels with *Aspergillus flavus* produced an average of 14.3 per cent virescent plants in the dry soil, and 51.0 per cent in the wet soil. *A. tamarii* produced, respectively, 7.5 and 48.6 per cent. Not only was there a decided numerical difference in virescent plants with respect to soil moisture, but chlorophyll deficiency per virescent plant was more pronounced in the damper soil. When considering only the plants that were more than half white, there were 1.6 per cent in the dry soil and 23.7 per cent in the damp soil. Adding more water immediately after planting, to bring the calculated soil moisture up to 35 per cent, had no additional effect on increasing virescence.

PREVIOUS SOIL CROPPING

In order to circumvent one of the uncertainties of securing virescent development, it was found necessary to use soils that had been stored 3 or more months without cropping. Whether the soil was kept damp or gradually allowed to dry in storage appeared to be immaterial. The soil was held at storage temperatures of 60° F. or warmer.

Soils brought from cropped land in the fall usually produced a less satisfactory virescence than did those that had been stored in a basement

from the previous year. Soil taken from different locations, however, varied considerably with respect to this reaction; but this difference disappeared after the soil had been in storage for some months. Soils taken from different cornfields in the fall gave just as much variation in results as did those that had been cropped with other plants.

While corn seedlings usually gave a good virescent reaction when grown on soils that had not been cropped for some months, yet, when the same soil was used for repeated tests in the greenhouse, it frequently gave poor virescence or nearly failed altogether in the third successive test. This was true regardless of whether Aspergillus inoculations had or had not been made in the previous tests. Sometimes, the previous cropping of soil with soybeans in the greenhouse also reduced virescence. In a test in late spring, when the temperature was too high for best virescent reaction, stored soil produced 30 per cent virescent plants, while cropped soils, different lots of which had just produced in the greenhouse mature crops of alfalfa, red clover, timothy, brome grass, and bluegrass, produced no virescent corn plants at all.

Attempts to improve the virescent reaction of corn on soils that were in a poorly reacting condition, by the addition of nitrates or complete fertilizer, or by sterilization with heat or formaldehyde, failed. Soil held in storage for several months, however, never failed. Attempts to decrease the virescent reaction of corn on stored soil by the liberal addition of chopped straw, well mixed with the soil a week before planting the inoculated corn, also was ineffective.

No satisfactory clue has yet been found as to the nature of the soil factors that have a marked influence on the development of induced virescence in corn seedlings.

SOIL TYPES

Dark silt loams, clay, fine gravel, greenhouse compost, blow sand and pure silica sand were all used successfully in producing induced virescence in corn seedlings grown from Aspergillus-inoculated seed. Little difference was noted, except that the chlorophyll-lacking areas appeared to be slightly more extensive in seedlings grown in soils rich in nitrates.

CAUSAL ORGANISMS

Aspergillus flavus and *A. tamarii* were found to cause induced virescence of corn seedlings under proper conditions. Ten different isolations of the former and 2 of the latter, isolated from several sources at different times, were used in the tests. All of them caused induced virescence, although some were slightly more effective than others. Two of the *A. flavus* isolations decidedly retarded the growth of the seedlings, but this gave them no advantage in the virescent reaction.

The following fungi, also used in inoculation tests on crown-injured corn, failed to cause induced virescence: *Aspergillus wentii, A. ochraceus, A. glaucus* (2 strains), *A. versicolor, A. niger, Penicillium notatum, P. palitans,*

P. viridicatum, P. expansum, P. oxalicum, Fusarium moniliforme (6 strains), *Diplodia zeae, Gibberella zeae*, and *Trichoderma* sp.

SPORE LOAD

Inoculations usually were made by soaking the kernels with broken seed coats for 1 or 2 minutes in spore suspensions containing about 10^7 spores per cc. Other spore concentrations with *Aspergillus flavus* also were tried. In one test spore concentrations of 10^7, 10^6, 10^5, and 10^4, produced, respectively, 37.3, 26.6, 26.7, and 24.8 per cent virescent plants. In another test, concentrations of 10^7, 10^5, and 10^3 produced, respectively, 69.7, 48.5, and 4.1 per cent.

ANTAGONISM OR COMPETITION

A number of fungi were used for inoculation simultaneously with *Aspergillus flavus* to test for possible effects from antagonism or competition. Inoculations were made in two concentrations; *A. flavus* spore suspensions of 10^7 and 10^5 spores, respectively, per cc. were mixed with an equal volume of 10^7 spores per cc. of the other fungus. Thus, in the latter case, there were approximately only 1/100 as many spores of *A. flavus* as of the other fungus. Adding *A. wentii, A. ochraceous, Penicillium palitans*, and *Trichoderma* sp. produced no appreciable effect on the virescent results when both cultures were at 10^7. *Aspergillus niger, P. viridicatum, P. notatum, P. expansum*, and *Fusarium moniliforme*, however, reduced virescence significantly under the same conditions. When *A. flavus* was used at a concentration of only 10^5, while the others were used at 10^7, all reduced virescence to half or less as compared with *A. flavus* at 10^5 alone, while *A. niger, P. viridicatum, P. expansum*, and *F. moniliforme* stopped the development of virescence entirely.

SEEDLING INVASION AND ROTS

Merely removing the seed coat from the crowns of corn kernels may allow decided pathologic effects in the consequent seedlings. In greenhouse tests with dark silt-loam soil taken from ordinary cropped fields near Urbana, the heights of seedling plants were checked and visible mesocotyl rots and internal mesocotyl infections were increased, (Table 1). The data are based on 4460 plants measured and blights observed, and 590 mesocotyls inspected and plated. When mesocotyl rots were found, they usually occurred near the lower end, adjoining the seed. Internal mesocotyl infection was determined by surface sterilizing for 1 minute with 1 : 1000 mercuric chloride and plating as previously described and illustrated (7, p. 77).

Seed treatment with suitable disinfectants largely prevented the pathologic effects that otherwise followed seed-coat injury (8).

Inoculating the seed with *Aspergillus flavus* had no noticeable outward effect on the seedlings when the seed coats were sound. A marked difference was observed, however, when the mesocotyls were plated, there being internal infection with this fungus in 18.8 per cent of the inoculated plants and only

0.6 per cent in the controls. When the seed coats had been removed from the crowns and the seeds inoculated with *A. flavus* before planting, the plants could be classified into 2 groups, *i.e.*, normally green and virescent. The normally green plants, grown from inoculated crown-injured seed, were nearly as vigorous as those grown from noninoculated crown-injured seed, but there was a significant increase in mesocotyl rots and internal mesocotyl infections in the former group. The virescent plants averaged shortest of all and had the highest percentage of mesocotyl infection.

There was nothing unusual about the mesocotyl rots being aggravated by seed inoculation with *Aspergillus flavus* or *A. tamarii*, or the occurrence of these fungi within the tissues of many apparently sound mesocotyls. Examination of young corn plants grown from seed inoculated with *A. niger*, *Penicillium oxalicum*, and *Trichoderma* sp. also revealed pronounced increases in mesocotyl rots and internal infections as compared with the checks. These latter fungi, however, caused no virescent reaction.

FIELD EXPERIMENTS

In field experiments conducted with crown-injured seed inoculated with *Aspergillus flavus* or *A. tamarii*, virescence developed abundantly in 3 years out of 5. In the other 2 years, virescence was rare. Coincidentally, soil moisture had been abundant prior to and for some days following planting time in the years when virescence occurred prominently, while the soil had been rather dry at planting time in the other 2 years. These results agree with those obtained in greenhouse experiments on the effect of soil moisture on the occurrence of induced virescence.

Inoculated corn, planted May 30, 1935, under excellent growing conditions following weeks of damp weather, produced 49 per cent virescent plants despite the long periods of daylight at that time of the year. The initial stands, as counted 12 days after planting, were very good: 95.8 to 92.3 per cent for the different lots. When recounted, 30 days after planting, the stand from good seed with sound seed coats had decreased 5.4 per cent, while similar seed, but with the seed coats removed from the crown, had decreased 11.8 per cent; and when the latter kind of seed had been inoculated with *Aspergillus*, the loss in stand below the original count was 18.9 per cent. A considerable part of the loss in stand in the last-named instance (where crown-injured seed had been inoculated) was caused by lack of sufficient chlorophyll for the plants to survive. Some other seedlings just managed to survive and produced very weak plants (Fig. 3). The average heights of the plants 30 days after planting were, respectively, for plants grown from kernels with sound seed coats not inoculated, injured seed coats not inoculated, and injured seed coats inoculated, 25.7, 17.5, and 14.1 inches. The variability in height within each group was the least in the first group and the greatest in the last. The acre yields from the same groups in the same year were 57.6, 39.8, and 28.8 bushels, respectively.

Dry weather conditions prevailed during the spring of the previous year (1934), there being only barely enough soil moisture for germination and growth at and for some time after planting on May 14. Virescence failed to develop from the inoculations. Field stands from the 3 groups: sound, non-inoculated seed coats; injured, noninoculated seed coats; and injured seed coats inoculated with *Aspergillus flavus*, were, respectively, 89.8, 89.0, and 88.9 per cent. Acre yields from the same 3 groups, respectively, were 41.6, 35.0, and 36.2 bushels. Thus, *A. flavus* inoculations under environmental conditions where virescence did not develop, failed to influence either the field stand or the yield of grain. Merely removing the seed coat from the crown, however, did significantly increase seedling mortality and decrease the yield of grain.

DISCUSSION

Heretofore, the term virescent seedlings has been used by other investigators only for the type that is directly controlled by hereditary genes. As the same kind of color effects can be produced in corn in which these hereditary genes are absent, the writers have been using the terms "hereditary virescence" and "induced virescence," to distinguish the 2 types.

Induced virescence, however, is governed also by hereditary factors, to a certain extent. Inbred lines can be produced that are highly resistant to this reaction, following inoculation with *Aspergillus flavus* or *A. tamarii*. Other inbreds, under the same conditions, are highly susceptible to induced virescence, although they contain none of the genetic factors directly responsible for virescence. All open-pollinated lots of corn, when tested under conditions suitable for the development of induced virescence, produced mixed populations of normal and virescent plants. Environmental factors, however, greatly influenced this reaction; so that the percentage of virescent plants might vary all the way from 0 to 95 in different tests of grain from the same ears.

Environmental conditions favorable to the development of a high percentage of virescent plants were also conducive to a greater chlorophyll deficiency per virescent plant. In other words, nearly white plants occurred only under conditions very favorable to incidence of virescence.

The development of induced virescence and genetic virescence is influenced by temperature and light in the same manner. In addition, however, induced virescence is influenced also by the soil complex. This complex may or may not be favorable to the interaction between host and parasite that results in induced virescence. As this type of virescence develops when pure quartz sand without the addition of nutrients is used as the substratum, it appears that the soil conditions in which virescence does not develop are of an inhibitory nature. The factors that bring about this inhibition are not yet understood. In numerous experiments the writers have so far not failed to put soil into proper condition for the development of induced virescence by first storing it in bins or covered metal cans for some months prior to

using and keeping the soil moisture moderately high while the corn is germinating. Soils taken from fields at planting time in the spring usually were satisfactory, but those taken in the fall after being cropped all summer often inhibited the development of induced virescence. Genetic virescence was not influenced in that way.

In both genetic and induced virescence, when the plants were in the 3-leaf stage, the fully green ones averaged somewhat taller than those deficient in chlorophyll. The difference in amount of chlorophyll no doubt was responsible for this. In addition, in the Aspergillus-inoculated plants, the virescent seedlings had somewhat more internal mesocotyl infection than those resistant to the virescent reaction (Table 1). This, however, may have been affected also, by chlorophyll deficiency. There appears to be no evidence that the virescent plants were virescent because of more intensive infection by *A. flavus* as compared with the similarly inoculated plants that developed normal green color. The principal reason for the difference in color reaction seems to be an inherited resistance of the plant, not to invasion by the fungus, but to the ability of the fungus to bring about the reaction that results in virescence.

The ultimate agent responsible for the virescent condition no doubt is the same whether the virescence be hereditary or induced. This is inferred not only because both types look alike but because both types seem to be influenced in the same way by temperature and light. The agent probably is of a chemical nature produced in one case by a chain of events initiated by the plant itself, when it carries certain hereditary genes, and in the other case similar results are produced by interaction of certain fungi with the seed or seedling.

SUMMARY

Corn-seedling virescence, indistinguishable from that caused by certain genetic factors, has been produced by inoculating the seed with either *Aspergillus flavus* or *A. tamarii*. To distinguish these 2 types of virescence with respect to their origin, the terms "genetic virescence" and "induced virescence" have been used.

In both types of virescence chlorophyll deficiency is aggravated by inadequate light and low temperature. Because a fungus, operating beneath the soil, is involved in the production of induced virescence, the soil condition, also, is important. Storing the soil, noncropped for several months before using, and keeping the soil moderately wet while the corn is being grown, were helpful in producing virescence. A number of widely different soil types and of different fertility levels gave somewhat similar results.

Open-pollinated corn varieties of the dent, sweet, pop, flint, and flour types developed induced virescence in a large number of the seedlings under proper conditions. A few highly resistant inbred lines of dent corn were found, but most inbred lines tested were susceptible. In order for the inoculation to be effective in producing virescence it was found necessary to break the seed coat or remove part of it. Shaving off the seed coat from the crowns

of the kernels with a sharp knife before shelling the ear was found to be very effective.

Ten isolations of *Aspergillus flavus* and 2 of *A. tamarii* from various sources were all effective in causing induced virescence, but there were differences in their potency. A number of other *Aspergillus* species and certain other fungi tested failed to give the virescent reaction.

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LITERATURE CITED

1. CARVER, W. A. A genetic study of certain chlorophyll deficiencies in maize. Genetics **12**: 415–440. 1927.
2. DEMEREC, M. Genetic relations of five factor pairs for virescent seedlings in maize. Cornell (New York) Agr. Exp. Stat. Mem. **84**. 38 pp. 1924.
3. EMERSON, R. A., G. W. BEADLE, and A. C. FRASER. A summary of linkage studies in maize. Cornell (New York) Agr. Exp. Stat. Mem. **180**. 83 pp. 1935.
4. EYSTER, W. H. Genetics of Zea mays. Bibliograph. Gen. **11**: 187–392. 1934.
5. Illinois Agr. Exp. Stat. A year's progress in solving farm problems of Illinois. Ill. Agr. Exp. Stat. Ann. Rept. **47**: 53–54. (1934). 1935.
6. Illinois Agr. Exp. Stat. A year's progress in solving farm problems of Illinois. Ill. Agr. Exp. Stat. Ann. Rept. **48**: 46–47. (1935). 1936.
7. KOEHLER, BENJAMIN, and J. R. HOLBERT. Corn diseases in Illinois. Ill. Agr. Exp. Stat. Bull. **354**: 74–75. 1930.
8. ———. Effect of seed coat injury on germination, vigor, and yield of corn. Trans. Ill. State Acad. Sci. **28**: 52–54. 1936.
9. LINDSTROM, E. W. Chlorophyll inheritance in maize. Cornell (New York) Agr. Exp. Stat. Mem. **13**. 68 pp. 1918.
10. PHIPPS, I. F. Inheritance and linkage relations of virescent seedlings in maize. Cornell (New York) Univ. Agr. Exp. Stat. Mem. 125. 63 pp. 1929.

EXPERIMENTS WITH LIQUID LIME SULPHUR FOR SPRAYING APPLES[1]

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(Accepted for publication May 16, 1938)

INTRODUCTION

From the time of the first use of liquid lime sulphur as a preventive spray for the control of apple scab (3) both experimenter and orchardist have been confronted with some aspect of the problem of spray injury to foliage and fruit. Following his experiments in 1907, Cordley (3) was originally led to recommend liquid lime sulphur, as a substitute for Bordeaux mixture because it seemed to give better scab control and, at the same time, to eliminate Bordeaux injury in the form of fruit russet. The following year, however, he noted a rather severe injury to the foliage following petal fall and also reports some dropping of fruit. As a result of these observations he

[1] Authorized for publication on February 9, 1938, as paper no. 824 in the the Journal Series of the Pennsylvania Agricultural Experiment Station, and as Contribution no. 117 from the Department of Botany.

[2] It is a pleasure to acknowledge our indebtedness to Henry Baugher in whose orchard this work was conducted, and also to J. Duain Moore and Donald Ayres for valuable assistance in recording the data in the field.

was led to use a slightly greater dilution of lime sulphur, and to recommend a 1–18 dilution in place of 1–15. The equal efficiency and greater safety of lime sulphur as compared to Bordeaux rapidly gained general recognition, yet the problem of spray injury has remained. It was early recognized by Wallace (7) that many factors could contribute to injury, such as the combination of lime sulphur with insecticides, the health and vigor of the tree, the method of application, as well as certain climatological factors at the time of and subsequent to the application.

Recent trends in spraying have included improved equipment, with much higher pressures, and an increased number of spray applications per season, both of which might be expected to increase injury. There has, however, been a corresponding reduction in strength of lime-sulphur solutions to the point where 1–40 or 1–50 has become almost a standard. Occasionally (1, 2), recommendations of 1–100 for summer applications have been made.

OBJECTIVES AND METHODS

In Pennsylvania, in our efforts to find a spray program that would provide a better balance between scab control and spray injury, we have been led to a further examination of the possibilities inherent in liquid lime sulphur. Some of our plots in 1935 and 1936 (5), (6) had shown that a 1–75 dilution of liquid lime sulphur produced distinctly less injury to foliage and fruit than the standard 1–50 dilution. During the season of 1937 it was our aim to study still further the effects of dilution. Some additional objectives included a study of the effects of 2 different dosages of each dilution selected, to which was added also a study of the effect of excess lime when added to each dilution and to each dosage, as well as a preliminary comparison of daytime *vs.* nighttime applications.

In order to facilitate statistical analysis of the results, the experiment as laid out involved 150 randomized single tree plots. There were 24 treatments and a nonsprayed check, each replicated 6 times. These plots were located in a solid block of Stayman trees, about 20 years old, in the orchard of Henry Baugher in Adams County, Pennsylvania. The various treatments are outlined in table 1.

In further explanation of table 1 it should be stated that all spraying was done with a single nozzle gun from the ground, each tree being covered first from underneath and, finally, from the outside. The random arrangement of the 6 individual trees to be covered with each material necessitated some extra driving. The use of a truck-mounted sprayer with a divided tank, however, made possible the use of 2 different materials at each filling, so that the extra driving did not prove to be a serious drawback. The sprayer was equipped with a 20-gal.-per-minute pump, and the pressure was maintained at 550 lbs. The guns as used to obtain the 2 different dosages were fitted with discs providing a 3/16 in. opening in the "big gun" and a 3/32 in. opening in the "small gun," with whirl plates to match, so that an equally fine spray could be obtained with either gun. In using these

TABLE 1.—*Plot treatment Baugher orchard sprayed in 1937 with liquid lime sulphur of different dilutions, amendments, and dosages*

Plot number	Dilution[a]	Amendment[b]	Dosage[c]
	Day applications throughout season		
1	1– 50 lime sulphur	no lime	big gun
2	1– 50 " "	" "	small gun
3	1– 50 " "	5–100 lime	big gun
4	1– 50 " "	5–100 lime	small gun
5	1– 75 lime sulphur	no lime	big gun
6	1– 75 " "	" "	small gun
7	1– 75 " "	5–100 lime	big gun
8	1– 75 " "	5–100 lime	small gun
9	1–100 lime sulphur	no lime	big gun
10	1–100 " "	" "	small gun
11	1–100 " "	5–100 lime	big gun
12	1–100 " "	5–100 lime	small gun
13	1–200 lime sulphur	no lime	big gun
14	1–200 " "	" "	small gun
15	1–200 " "	5–100 lime	big gun
16	1–200 " "	5–100 lime	small gun
	Day applications in early sprays Night applications beginning with first cover spray		
17	1– 75 lime sulphur	no lime	big gun
18	1– 75 " "	" "	small gun
19	1– 75 " "	5–100 lime	big gun
20	1– 75 " "	5–100 lime	small gun
21	1–100 lime sulphur	no lime	big gun
22	1–100 " "	" "	small gun
23	1–100 " "	5–100 lime	big gun
24	1–100 " "	5–100 lime	small gun
25	Check—no sprays		

[a] Dilutions based on a specific gravity reading of 1.30 for the concentrated lime sulphur.

[b] Lead arsenate at 3 lb. per 100 gal. was used in all applications. Fresh hydrated lime was used in the plots indicated beginning with the petal fall and in all subsequent applications.

[c] Variations in dosage with the first cover spray. Use of the "big gun" resulted in applying 12 gal. per tree per application, while "small gun" applied 6 gal. per tree per application. Previous to the first cover spray the "big gun" was used on all plots.

guns an effort was made to cover each tree in a uniform manner. The dosage as applied by the "big gun" amounted to 12 gal. per tree and by the "small gun" to six gal.

The dates of the various spray applications to the Baugher orchard in 1937 were as follows:

Delayed dormant, April 17; Prepink, not applied; Pink, May 3; Petal Fall, May 19; First Cover, June 1; Second Cover, June 11 and 12; Third Cover, June 23; Fourth Cover, July 16–18.

It was not intended that the prepink application should be omitted, but, at that time, the orchard was so wet that it was impossible to drive through it.

Scab was first noticed on the leaves of the check trees on May 12. In the first week in June some preliminary leaf counts were made showing 3 per cent scab where the 1–50 dilution had been used, 7 per cent at 1–75,

9 per cent at 1–100 and 13 per cent at 1–200, with 49 per cent on nonsprayed checks. At this time there was no noticeable effect either from the use of lime or the variation in dosage, since only the first cover spray had been applied.

As the season progressed other differences began to appear. The trees sprayed with 1–200 lime sulphur without lime showed severe foliage burning. This appeared to be of the type generally ascribed to free arsenic in the spray mixture. Accompanying the injury there was a definite "burn out" of the early scab spots. During the week of July 7–15, just prior to the fourth and last cover spray, detailed counts of scab and leaf injury were made as follows: Five hundred leaves from each of the 150 trees, 75,000 in all were counted for scab, and an additional 500 from each tree were counted for injury. An effort was made to count a true random sample from each tree by selecting terminals and spurs, some from the top, some from the inside, and some from around the outside that could be reached from the ground. The counting was done by 2 people, one working only on scab and the other only on injury. Leaves were recorded as scabby or clean, injured or uninjured; burned out scab spots showing no evidence that the fungus was still alive were counted as clean at that time. No effort was made to record slight and severe injury separately, but simply to get an accurate figure for percentage of foliage injured. The mean percentages of scabby leaves for each treatment, *e.g.*, average of 6 trees or 3,000 leaves is given in table 2. Since scab on the foliage is but an indication of what may be expected on the picked fruit, no effort was made to analyze these figures further. Certain facts, however, seem to be self-evident. The large dosage, as represented by the "big gun" gave better control than the small dosage, in every instance but one. Lime apparently had little effect except in the 1–200 dilution where it was effective in preventing the burn out of scab. A similar effect seems evident in the 1–75 dilution at night, although it is not apparent in the 1–100 dilution at night, a fact for which we have no explanation at present.

TABLE 2.—*Percentage of leaves showing scab in Baugher orchard, July 7–15, 1937*

Dilution	Without lime		With lime 5 lb.–100 gallons	
	Big gun	Small gun	Big gun	Small gun
1– 50 day	4.2[a]	5.2	3.8	4.2
1– 75 day	5.4	5.2	5.6	7.8
1–100 day	6.4	9.8	6.5	9.9
1–200 day	1.3	4.8	10.8	17.5
1– 75 night	2.7	5.1	7.1	7.9
1–100 night	9.9	11.3	7.5	8.7

[a] Each percentage figure represents the average of 3000 leaves, or 500 leaves from each of 6 replicates.

The data on percentage of leaves showing injury, number of scabby apples, and yield are presented in their entirety in table 3. These data

TABLE 3.—*Detailed data on spraying experiments*

Treatment	Percentage of leaves showing injury[a]							Number of scabby apples[b]							Yield: bushels per tree						
	1	2	3	4	5	6	Mean	1	2	3	4	5	6	Mean	1	2	3	4	5	6	Mean
1	27.1	20.1	31.8	21.1	29.9	28.9	26.5	7	9	9	1	14	4	7.33	8.1	8.0	12.5	6.0	14.0	7.0	9.2
2	14.7	19.4	23.8	15.6	20.9	19.1	18.9	9	4	7	7	8	19	9.00	15.0	9.0	9.0	11.9	11.0	9.0	10.8
3	27.2	14.1	29.7	29.9	25.7	26.5	25.5	7	0	5	1	6	19	6.30	8.2	6.6	6.0	8.0	7.7	9.0	7.6
4	20.7	17.5	24.7	18.5	28.0	20.3	21.6	11	15	4	17	9	17	12.16	8.9	9.0	10.0	18.0	6.0	14.0	10.9
5	19.7	30.1	15.1	20.0	18.7	30.9	22.4	9	12	11	21	26	13	15.33	8.3	10.0	13.0	12.6	9.0	8.0	10.1
6	6.2	23.7	13.1	16.0	23.7	18.2	16.8	16	8	17	5	5	53	17.33	14.3	10.0	18.0	13.0	10.0	15.5	13.4
7	15.3	16.3	20.4	16.8	18.8	12.7	16.7	14	13	18	9	13	12	13.16	14.0	21.0	18.0	14.0	11.6	16.0	15.8
8	26.1	16.2	15.7	15.1	13.5	10.0	16.1	15	19	78	17	22	20	28.5	14.0	16.0	22.0	11.2	14.0	12.5	14.9
9	14.5	14.6	31.0	16.7	13.7	18.0	18.1	16	20	8	24	31	18	19.50	9.2	17.0	11.0	17.5	19.0	6.0	13.3
10	6.4	12.4	16.4	14.0	19.9	13.5	13.8	29	42	42	27	38	52	38.33	13.7	21.0	11.0	11.4	6.0	15.0	13.0
11	20.8	14.0	12.3	14.1	11.6	15.5	14.7	47	28	49	19	26	14	30.50	9.5	14.0	19.0	16.0	12.0	9.3	13.6
12	14.2	12.1	15.5	21.4	12.3	13.6	14.8	14	30	26	52	57	67	41.00	7.5	14.0	8.0	6.9	15.0	16.0	11.2
13	79.0	87.1	90.4	83.6	82.9	82.6	84.3	51	28	22	26	25	28	30.00	14.0	7.0	7.0	5.0	7.2	8.0	8.0
14	70.3	73.6	67.2	49.6	78.3	75.7	69.1	60	86	77	72	36	39	61.66	10.9	13.0	12.0	10.0	12.0	14.0	11.9
15	17.3	15.4	5.2	12.3	5.9	6.1	10.4	26	43	100	25	64	28	47.66	13.5	13.0	20.6	5.8	18.8	7.0	13.1
16	7.7	9.3	9.9	8.2	12.2	15.0	10.4	84	57	76	102	117	34	78.33	7.5	16.0	14.0	13.0	17.0	4.8	12.0
17	48.6	48.5	51.8	38.9	55.3	42.9	47.7	13	20	4	8	5	2	8.66	12.2	9.0	4.0	11.0	6.9	6.0	8.2
18	35.6	28.0	23.5	37.8	31.2	45.4	33.6	37	14	21	4	22	13	18.51	16.6	9.0	18.0	11.6	14.0	5.0	12.3
19	16.7	18.5	17.7	10.0	16.6	20.0	16.6	17	22	10	18	5	21	15.5	12.0	14.0	15.0	15.8	6.5	9.0	12.0
20	17.0	10.9	19.3	11.8	13.3	6.7	13.2	33	48	24	43	42	48	39.66	11.0	21.0	8.0	15.0	18.0	20.0	15.5
21	12.9	16.2	24.6	20.5	24.2	15.0	18.9	38	50	22	29	33	51	37.16	16.2	19.0	18.0	9.0	8.6	20.0	15.1
22	21.3	18.8	16.5	23.0	21.2	25.4	21.0	65	39	64	28	45	48	48.16	18.0	20.3	20.7	9.5	15.2	16.7	16.7
23	13.2	22.3	13.7	12.6	12.6	14.8	14.9	27	15	15	10	28	44	23.16	12.1	10.0	14.0	14.2	17.0	21.2	14.7
24	6.2	18.0	15.7	11.3	16.6	10.8	13.1	62	56	14	15	34	61	40.33	16.0	12.6	9.0	16.0	13.0	13.4	13.3
25	5.6	5.9	6.0	6.5	10.3	6.4	6.8								1.8	2.0	4.0	6.9	0.0	2.0	2.8

[a] Each percentage is based on a count of approximately 500 leaves. (See text.)
[b] Numbers are based on samples of 300 apples, percentage figures are readily obtained by dividing each number by 3.

TABLE 4.—*Analysis of variance of data presented in table 3*

Analyses and interactions	Foliage injury			Scab on fruit			Yield		
	Degrees of freedom	Sum of squares of deviations	Mean square (variance)	Degrees of freedom	Sum of squares of deviations	Mean square (variance)	Degrees of freedom	Sum of squares of deviations	Mean square (variance)
Preliminary Analysis									
Total	149	50,603.19		143	74,457.23		143	2,719.94	
Treatments	24	47,446.64	1,986.94	23	46,964.89	2,041.95	23	869.53	37.80
Replicates	5	152.19	30.44	5	635.23	127.04	5	60.88	12.17
Remainder	120	3,004.36	25.04	115	26,857.11	233.54	115	1,789.53	15.56
Analysis									
Total	143	48,855.30		143	74,457		143	2,719.94	
Dilutions (D)	5	13,349.28	2,669.85	5	31,555	6,311	5	308.98	61.79
Lime (L)	1	10,309.01	10,309.01	1	1,066	1,066	1	28.09	28.09
Dosage (G)	1	732.64	732.64	1	7,980	7,980	1	62.53	62.53
Interactions									
D × G	5	351.91	70.38	5	2,598	520	5	191.66	38.33
D × L	5	20,345.66	4,069.13	5	3,002	600	5	292.43	58.58
L × G	1	308.80	306.80	1	207	207	1	60.30	60.30
Remainder	125	3,463.00	27.7	125	28,049	224.39	125	1,775.93	14.20

Least significant difference between means of Dilutions = 3 per cent.
Least significant difference between means (lime *vs.* no lime) = 0.5 per cent.
Least significant difference between means Dosages (''big gun'' *vs.* ''small gun'') = 0.5 per cent.

Least significant difference between means of dilutions = 8.56 apples or 2.85 per cent.
Least significant difference between means of lime *vs.* no lime = 4.94 apples or 1.65 per cent.
Least significant difference between means of Dosage (''big gun'' *vs.* ''small gun'') = 4.94 apples or 1.65 per cent.

Least significant difference between means of dilutions = 2.15 bu.
Least significant difference between means of lime *vs.* no lime = 0.3 bu.
Least significant difference between means of Dosage (''big gun'' *vs.* ''small gun'') = 0.3 bu.

are presented in order to show the amount of variability present in such an experiment, even in a block of trees especially selected for uniformity. An examination of the table will reveal the necessity for statistical analysis in order to determine the significance of comparatively small differences in average percentages.

The 3 sets of data presented in table 3 were subjected to analysis for variance. These analyses are shown in table 4. In each case a preliminary analysis of the data is necessary in order to judge whether significance can be attached to differences between treatments or between the replications or both. From the preliminary analyses it is at once evident that no significance is to be attached to differences between replicates, but that there is a high degree of significance among the treatments. Since the treatments themselves involved 3 variable factors, *i.e.*, dilution, dosage, and lime, some further analysis of the data is desirable in order to determine how much significance may be attached to each separate variable. Omitting the non-sprayed trees as irrelevant to any further analysis and omitting also the replications, already shown to have no significance, and considering the night-sprayed plots simply as additional dilutions, we are able to make another analysis on the basis of 3 criteria of classification, with results as shown further in table 4.

TABLE 5.—*The effects of dilution, dosage and lime on leaf injury and fruit scab, and yield of fruit (comparison of effects of dilutions)*

Dilution	Average leaf injury	Average fruit scabbed	Average yield per tree
	Per cent	*Per cent*	*Bu.*
1– 50 day	23.1	2.9	9.6
1– 75 day	18.0	6.2	14.0
1–100 day	15.3	10.8	12.8
1–200 day	43.5	18.1	11.3
1– 75 night	27.7	6.9	12.0
1–100 night	16.9	12.4	14.8
Check	6.8	86	2.8
Significant difference	3.0	2.85	2.15

In table 5 are shown the mean percentages of leaf injury and fruit scab and mean yield per tree, as influenced by the dilution of the sprays applied. From this table it is evident that the 1–50 dilution produced less fruit, less scab, and more foliage injury than did the 1–75 dilution and that these differences in each case are highly significant. The amount of leaf injury decreases as the dilution increases until a dilution of 1–100 is reached, beyond which point the leaf injury is greatly increased. It seems evident from the work of Hodgkiss and Frear (4) that the effect here is attributable to the liberation of soluble arsenic. The percentage of scabby fruit shows a very consistent relation to the dilution. The differences here shown indicate a very significant increase in scab with each corresponding increase in dilution. It will be recalled that scab on the foliage behaved in a similar fashion

(Table 3). This being true, it seems apparent that if lime sulphur be diluted for the sake of safety, then scab control rapidly becomes a limiting factor. In the case of yield, the results are not so clear cut; yet, it seems certain that both extremes of dilution have resulted in decreasing the yield. So far as night spraying is concerned, there was no significant effect on scab control. At a dilution of 1–75, spraying at night produced more injury than did the same dilution applied in the daytime; yet, with the 1–100 dilution, this effect was not so noticeable. These differences in injury appear also to be correlated with similar differences in yield.

TABLE 6.—*Comparison of effects of dilution vs. dosage*

Dilution	Percentage leaf injury			Percentage fruit scab			Yield, bu. per tree		
	Big gun	Small gun	Diff. in favor big gun	Big gun	Small gun	Diff. in favor big gun	Big gun	Small gun	Diff. in favor big gun
1– 50 day	26.0	20.2	– 5.8	2.28	3.52	1.24	8.4	10.9	– 2.5
1– 75 day	19.5	16.4	– 3.1	4.75	7.63	2.88	12.9	14.2	– 1.3
1–100 day	16.4	14.3	– 2.1	8.33	13.22	4.89	13.3	12.1	+ 1.2
1–200 day	47.3	39.7	– 7.6	12.94	23.33	10.39	10.6	12.0	– 1.4
1– 75 night	32.1	23.3	– 8.8	4.02	9.69	5.67	10.1	13.9	– 3.8
1–100 night	16.9	17.0	+ 0.1	10.05	14.75	4.73	14.4	15.0	– 0.6
Average	26.4	21.8	*– 4.6*	7.06	12.20	*4.96*	11.6	13.0	*– 0.6*
	[a] Significant difference = 0.5			Significant difference = 1.65			Significant difference = 0.3		

[a] Figures for significant differences apply only to the differences between the averages. Further analysis of covariance would be necessary to determine the significance of the individual differences at the several dilutions.

In table 6 it can be readily seen that the larger dosage in the cover sprays, as represented by the "big gun" was productive of greater foliage injury and better scab control. The correlation between dosage and dilution is very marked in both cases. It also is apparent that both these factors find

TABLE 7.—*Comparison of effects of dilution vs. lime*

Dilution	Percentage leaf injury			Percentage fruit scab			Yield, bu. per tree		
	Lime	No lime	Diff. in favor lime	Lime	No lime	Diff. in favor lime	Lime	No lime	Diff. in favor lime
1– 50 day	23.5	22.7	– 0.8	3.1	2.7	– 0.4	9.3	10.0	– 0.7
1– 75 day	16.4	19.6	3.2	6.9	5.4	– 1.5	15.3	11.8	3.5
1–100 day	14.7	15.9	1.2	11.9	9.6	– 2.3	12.3	13.1	– 0.8
1–200 day	10.4	76.7	66.3	21.0	15.3	– 5.7	12.6	10.0	2.6
1– 75 night	14.7	40.6	25.9	9.6	4.5	– 5.1	13.8	10.3	3.5
1–100 night	14.0	19.9	5.9	10.6	14.1	3.5	14.1	15.9	– 1.8
Average	15.6	32.2	*16.6*	10.5	8.6	*– 1.9*	12.9	11.8	*1.1*
	[a] Significant difference = 0.5 per cent			Significant difference = 1.65 per cent			Significant difference = 0.3 bu.		

[a] Figures for significant differences apply only to the differences between the averages. Further analysis of covariance would be necessary to determine the significance of the individual differences at the several dilutions.

additional expression in terms of yield. Here, it would seem, spraying was more detrimental than beneficial unless we keep in mind the performance of nonsprayed treees.

Turning next to the effects of lime when added to the various dilutions as summarized in table 7, it is at once evident that lime has a very beneficial effect, so far as reducing injury is concerned. This effect is most pronounced in the extreme dilutions. That it also permits a greater build up of scab is readily recognized, and that, in general, it has produced a better yield seems not to be denied.

DISCUSSION

The field experiment in orchard spraying here outlined is, first of all, illustrative of methods that, it is believed, have been all too little used by plant pathologists interested in the control of fruit diseases. The senior writer first used this type of experimental procedure in his own work in 1935 (5), and has long felt that in experimental field work involving so many controversial points as does orchard spraying, the investigator can ill afford to leave any stone unturned that might contribute to his efforts to secure accurate, unbiased, quantitative information. Following the season of 1937, we are more than ever convinced that a randomized-plot layout, such as we have here employed, can be used to advantage and that the extra driving and labor involved in the physical application of materials are worth the effort.

No problem so complicated as orchard spraying, involving as it does an attempt to balance disease control against possible injury, can be solved in one year's field work. Indeed, it is possible in view of the ever increasing list of new materials, new equipment, ever-changing seasonal conditions, etc., that it may never be solved to the satisfaction of either grower or investigator. Yet, at the same time, progress can be made and should be recorded in such manner as to build an ever-firmer foundation for field practices.

It should, perhaps, be pointed out that the data on foliage injury do not give a complete picture of the injury. While the percentage of injured foliage has been recorded, the relative areas of injured leaf surface are not apparent from the figures. Thus the injury recorded for the 1–200 dilution without lime, was actually more severe than even the high percentage of injured leaves would indicate, on account of the large area of leaf surface involved. Conversely, the 1–75 dilution actually appeared in the field to much better advantage than the percentage figures would indicate because, for most of the leaves recorded as injured, the amount of leaf surface involved was relatively very small. It would have been of interest and value to record such injury in terms of the relative sizes of the injured areas, but time and labor at our disposal did not permit such detailed records in this instance.

While we are not here concerned primarily with recommendations, it does seem evident that a lime-sulphur dilution of approximately 1–75, if used throughout the season, offers a better chance of adequate scab control, together with a minimum injury, than either a stronger or a weaker solution. While the stronger solution gives better scab protection, and the weaker solution with additional lime may be slightly safer, the 1–75 dilution provides a better balance when both control and injury are considered.

SUMMARY

Studies in orchard spraying are described that make use of replicated, randomized single tree plots, and permit analysis of variance of the results.

These studies deal with dilutions of liquid lime sulphur, together with such factors as dosage and the addition of hydrated lime, and the effects of these upon foliage injury, fruit scab and tree yield.

Scab has been shown to increase progressively with the dilution. Foliage injury, on the other hand, shows a tendency to decrease with increased dilution up to 1–100, and to increase sharply beyond that point.

Of two dosages used, the larger resulted in greater injury and better scab control, but decreased yield.

The use of lime with the various dilutions and dosages is shown to reduce injury and increase yield, but to permit a slightly greater development of scab.

When foliage injury, scab and yield are all considered a dilution of 1–75 provides the best balance between injury and scab control.

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LITERATURE CITED

1. Anonymous. Fifty-third annual report of the Ohio State Agricultural Experiment Station. Ohio Agr. Exp. Stat. Bull. 548: 48. 1935.
2. ————————. Spraying program and pest control for fruit crops. Ohio Agr. Exp. Stat. Bull. 562. 1936.
3. CORDLEY, A. B. Lime sulphur spray to prevent apple scab. Better fruit 3(3): 26; (10): 33–34. 1908.
4. HODGKISS, W. S., FREAR, D. E. H. and WORTHLEY, H. N. A chemical study of mixtures of lime sulphur and lead arsenate. Journ. Econ. Ent. 31: 443–455. 1938.
5. THURSTON, H. W., JR. and H. N. WORTHLEY. Some problems in apple spraying in Pennsylvania. Penn. Agr. Exp. Stat. Bull. 324: 19. 1936.
6. ————————. Spray materials in relation to spray injury. Proc. State Hort. Assoc. Penn. 78: 72–81. 1937.
7. WALLACE, E. Spray injury induced by lime-sulphur preparations. New York (Cornell) Agr. Exp. Stat. Bull. 288: 103–137. 1910.

SUSCEPTIBILITY OF NEEDLES OF DIFFERENT AGES ON PINUS MONTICOLA SEEDLINGS TO CRONARTIUM RIBICOLA INFECTION

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(Accepted for publication July 23, 1938)

REVIEW OF PREVIOUS STUDIES OF PINUS STROBUS AND P. MONTICOLA

Since Clinton and McCormick first demonstrated in 1917 that white pine blister rust (*Cronartium ribicola* Fischer) enters its aecial host through the needles (3), considerable interest has been evidenced as to possible differences in susceptibility between needles of various ages and the influence of such differences on the cankering of pines. From their work on eastern white pine, *Pinus strobus* L., Clinton and McCormick concluded, although positive evidence was lacking, that the sporidial germ tubes gain entrance into the needles through the stomata (3, p. 449). Accepting this conclusion, Spaulding (14) attempted a possible explanation of the differences in susceptibility between species of white pine on the basis of needle persistence and distribution of stomata. In a recent publication by Hirt (5), it is shown that the sporidial germ tubes are capable of penetrating directly through the epidermis and it is suggested that this, as is true of most other Uredinales (1, p. 229; 2, p. 257), may be the usual mode of entrance.

The results of studies on eastern white pine have not been entirely consistent, but, in general, have shown needles of the current and second seasons to be about equal in susceptibility. In some cases, however, there are indications that needles of the current season may be slightly more susceptible. Differences in methods employed by investigators may partly explain the lack of uniformity in results.

Various methods of inoculation employed by Clinton and McCormick in 1917 and 1918 consistently resulted in the highest percentage of infection on 1-year-old pines (3, p. 445).

In 1921 York and Snell (15) were successful in inoculating potted seedlings of *Pinus strobus* of the seasons 1918 and 1921. A fascicle of needles from a 1918 seedling and one entire 1921 seedling were sent to Dr. R. H. Colley for critical examination. He reported to them, in part: "If anything, there are proportionately more individual infections in the needles of the 1921 seedling than in full grown leaves."

Inoculations made by Snell and Gravatt in 1922 (12, p. 589) were as successful on 2-year-old needles of *Pinus strobus* as on needles but 1 year old.

In a later paper (16), the above 1921 and 1922 inoculations are discussed further. In comparable 1921 experiments 77 per cent of the year-old seedlings became infected, whereas only 58 per cent of the older ones (mostly over 3 years old) became infected (p. 502). In the 1922 inoculation of potted pines, seedlings of all ages except year-old ones became infected (p.

505). Quoting directly from York *et al.* (16, p. 507): "Clinton (2) and the writers (7, 17) stated that both the first and second year needles of *Pinus strobus* may become infected, but they gave no data on the relative susceptibility of needles of different ages. In an attempt to get some definite information on this point in 1922, 60 inoculations were made on needles of known ages on older trees. Two per cent of these inoculations were made on 1920 needles, 18 per cent on 1921 needles, and 80 per cent on 1922 needles. None of the inoculations on 1920 needles, 18 per cent of those on the 1921 needles, and 19 per cent of those on the 1922 needles developed leaf spots. None of the inoculations on 1920 needles, 9 per cent of those on 1921 needles, and 8 per cent of those on the 1922 needles resulted in twig cankers. These results agree with the statement previously made that 1921 and 1922 needles seemed of approximately equal susceptibility (7)."

From observations made in 1920 Richards (9) concluded that the relative susceptibility of eastern-white-pine needles to blister rust infection increases with the age of the needles. It must be realized, however, that the exact date of infection was impossible to determine.

From an analysis of 15,000 cankers in the Adirondacks, Snell (10, p. 479) reasoned that in all probability many cankers originate on 2- and 3-year-old needles as well as on those of the current season.

In 1927 Hirt (4) exposed 3-year-old transplants of *Pinus strobus* to sporidial infection on *Ribes nigrum* L. for a 24-hour period before removal to a permanent planting area. Upon the trees that became infected as a result of this exposure over 900 cankers developed, approximately two-thirds of which appeared on 1926 wood and the remainder on 1927 wood.

Snell (11) reports, for the first time, the results of two series of inoculation experiments on *Pinus strobus* that yielded contradictory results, one showing the current-season needles to be higher and the other showing them to be lower in susceptibility than the older needles. In this same paper he summarizes previously published evidence from which he concludes, in effect, that the current-season needles of *P. strobus* are to a greater or less degree more susceptible to *Cronartium ribicola* than are the second-season needles, although he does not consider the question incontrovertibly settled.

The results of studies conducted in a manner similar to that employed by the authors have recently been published by Hirt (5). Working with *Pinus strobus* he found that the current-season needles are only about one-fourth as susceptible as year-old needles (p. 189).

Needle susceptibility data for western white pine, *Pinus monticola* Dougl., have not been previously determined by precise inoculation experiments. From an analysis of approximately 6,000 cankers on both large and small trees, however, Lachmund (6, 7) offers very convincing evidence that the current-season needles of this species are relatively resistant to blister-rust infection. In one of these papers Lachmund (6) stresses the importance of this resistance in studies wherein it is desirable to determine the year of origin of established waves of pine infection.

In 1933, inoculation experiments were conducted near Bovill, Idaho, with small potted western white pines. This paper describes these experiments, which were conducted as a check on the validity of Lachmund's (7, p. 920) previous conclusion that the current-season needles of *Pinus monticola* are relatively resistant to infection by *Cronartium ribicola*.

TEST PLANTS[1]

Forty-six thrifty *Pinus monticola* seedlings from 5 to 7 years of age and from 7 to 15 inches in height were selected as test material from a presumably rust-free area near Bovill, Idaho, on September 7, 1933. Careful examination of these trees had failed to disclose any cankers or any needle spots that might have been blister-rust infections. Fall needle cast was complete on all trees and the remaining needles gave evidence of remaining attached for the duration of the study. The trees were carefully dug and then transported in wet moss to Deep Creek, near the town of Elk River, Idaho, where they were immediately potted in native forest soil.

Thirty-four of these potted pines were exposed to infection by *Cronartium ribicola* in the manner described in the following section. The 12 remaining trees served as check plants and were transported back to the Bovill area immediately after potting and before being exposed to infection.

EXPOSURE OF PINES TO INFECTION

On September 7, 1933, the 34 potted pines were placed in trenches dug in the moist sand bordering Deep Creek. Dense concentrations of native *Ribes petiolare* Dougl., a common stream-type species of high susceptibility and telium-producing capacity (8), formed an almost closed leafy canopy over the entrenched pines. The lower leaves on the bushes were bearing very heavy blister-rust infection in the telial stage. The uppermost leaves bore well-advanced uredial infection that showed promise of soon developing telia, thus insuring a continual supply of viable sporidia to the pines.

Rainfall occurred intermittently over the white-pine belt of northern Idaho between September 9 and 24, inclusive. The experiment at Deep Creek was being conducted in the bottom of a damp, well-shaded canyon, making conditions for sporidial production and pine infection favorable throughout the entire 16-day period. Climatic conditions after September 24 were not so favorable for pine infection. Severe frosts occurred in the last week of September and when visited on October 4 the *Ribes* plants surrounding the potted pines were completely defoliated.

On October 11 the 34 exposed pines and the 12 check plants left near Bovill were removed to a greenhouse[2] at Moscow, Idaho. The 34 pines, then, had been in close proximity to infected *Ribes* for a total of 35 days (Sept. 7 to Oct. 11). Spaulding (13) has shown, for certain other *Ribes* species, that when plants are defoliated by autumnal frost the telia may remain viable for

[1] The pines used in these experiments were made available through the courtesy of the Office of Blister Rust Control, Spokane, Washington.

[2] Greenhouse and laboratory space were provided through the courtesy of the School of Forestry, University of Idaho.

several weeks on the fallen leaves. In view of his findings it seems logical to assume that the pines in this study were exposed to infection throughout the entire 35-day period, although there were no leaves on the *Ribes* plants after October 4. During only 16 of these days (Sept. 9 to 24), however, were climatic conditions generally favorable for pine infection.

CARE AND EXAMINATION OF PINES

Once in the greenhouse, the group of 34 exposed pines and 6 of the check plants were kept at a temperature nearly the same as that outside. The 6 remaining check plants were left in a cold frame out-of-doors. Careful observations were made daily in order to detect the first visible evidences of needle infection. The first minute needle spots became visible on November 9, 1933, and from then until November 16 hundreds of spots appeared daily on all ages of needles. One month was allowed, thereafter, for further incubation and an analysis of the infection was then made. Thus 66 days had elapsed since the pines were last exposed to infection, allowing ample time for at least the majority of the infection spots to become visible on needles of all ages. Clinton and McCormick (3, p. 442) found that needle spots become visible to the naked eye a month or two after inoculation.

When the first rust spots began to appear on the needles of the exposed seedlings identification was made positive by microscopic examination. Characteristic blister-rust mycelium (3, pp. 450–451) was found in abundance within the needle tissues, particularly in the mesophyll area. Later, as the needle spots acquired their characteristic appearance and translucent lemon-yellow color (3, plate 40; 16, plate 1), identification was made macroscopically and occasionally verified microscopically. An examination of the 12 check plants failed to reveal any needle spots that could be identified as white-pine blister-rust infections.

In view of the variation encountered in western-white-pine needles it was considered most accurate to present the final susceptibility data on a basis of needle area. Needle area was computed from the formula: $\text{Area} = N \times L (C + 10r)$, in which N = number of fascicles on a given internode, L = length of needles, C = circumference of fascicle at the base, and r = radius of fascicle. The relation of C and r to length of needle fascicle was determined from measurements of 355 fascicles collected on seedlings from the locality where the test plants had been secured. In computing area of foliage on the test plants it was only necessary to measure fascicle length and then enter that value and the corresponding C and r values in the formula. No allowance was made for the tapering of the needles.

RESULTS

Table 1 shows the relative susceptibility of needles of different ages to white-pine blister rust in terms of number of infection spots per 100 square centimeters of foliage surface exposed. From this table it is strikingly evident that the current-season needles are decidedly lower in susceptibility than are the older needles. This relationship holds true irrespective of length

TABLE 1.—*Needle spots developed on potted 5- to 7-year-old Pinus monticola seedlings from exposure to infection by Cronartium ribicola on native Ribes petiolare from September 7 to October 11, 1933*

Length of time needles retained	Trees	Year's wood bearing needles	Needles	Surface area of needles		Needle spots		Needle spots per 100 square centimeters of surface area
				Actual	Per cent of total	Actual	Per cent of total	
(*Years*)	(*No.*)		(*No.*)	(*Sq. cm.*)		(*No.*)		(*No.*)
		1933	895	941	51.4	8	4.0	.9
4	4	1932	685	401	21.9	67	33.7	16.7
		1931	410	433	23.6	117	58.8	27.1
		1930	79	57	3.1	7	3.5	12.4
		1933	7,390	7,835	55.0	90	7.2	1.2
3	20	1932	4,445	5,162	36.3	956	77.0	18.5
		1931	938	1,236	8.7	196	15.8	15.9
		1933	1,940	2,348	59.0	48	12.3	2.0
2	10	1932	1,405	1,649	41.0	341	87.7	20.7
	34	1933	10,225	11,124	55.4	146	8.0	1.3
Totals and	34	1932	6,535	7,212	36.0	1,364	74.5	18.9
averages	24	1931	1,348	1,668	8.3	313	17.1	18.8
	4	1930	79	57	.3	7	.4	12.4

of time the needles are retained. Combining the results for all trees tested, needles of the second and third seasons are found to be essentially equal in susceptibility. Fourth-season needles, although poorly represented, are somewhat lower in susceptibility than second- and third-season needles but decidedly higher than current-season needles.

Although the exact date of infection resulting in a given needle spot could not be determined the minimum incubation period was not less than 29 days (last possible date of infection Oct. 11; first needle spot appeared Nov. 9). The most probable incubation period, however, was between 46 and 68 days. (Most favorable infection period Sept. 9 to 24; majority of needle spots appeared Nov. 9 to 16).

For the complete picture from needle infection to canker development it would be necessary to know the number of fascicles bearing infected needles on internodes of various ages and also just how many of these infected fascicles remain attached sufficiently long to permit the fungus mycelium to grow down the needle and become established in the bark of the twig. It must be kept in mind that the study herein reported was concerned only with the relative susceptibility of needles of different ages and that no consideration was given to subsequent canker development. The salient feature of this study was the determination that needles of the current season are relatively low in susceptibility to infection by white-pine blister rust and that needles of the second and third seasons are relatively high in susceptibility. Lachmund's scale of canker distribution (6, pp. 689–690, 692) shows that relatively few cankers form on internodes of the current season, whereas the majority form on internodes of the second and third seasons. The relative susceptibility of needles of different ages corresponds, in general, to the relative abundance of cankers formed on internodes of different ages. From this comparison it seems logical to conclude that needle susceptibility is a major factor governing the distribution of cankers on internodes of various ages.

SUMMARY

Thirty-four potted native *Pinus monticola* seedlings ranging from 5 to 7 years of age and from 7 to 15 inches in height were exposed to *Cronartium ribicola* infection on native *Ribes petiolare* bushes from September 7 to October 11, 1933, at Deep Creek, Idaho. After a minimum incubation period of 66 days the resultant needle spots were tallied and summarized on the basis of age of needles exposed. From this tally it was apparent that needles of the current season are relatively low in susceptibility, needles of the second and third seasons relatively high and approximately equal in susceptibility, and needles of the fourth season intermediate but still quite high in susceptibility.

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OF FOREST PATHOLOGY, BUREAU OF PLANT INDUSTRY.
MAINTAINED AT PORTLAND, OREGON, IN COOPERATION
WITH FOREST SERVICE, UNITED STATES
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LITERATURE CITED

1. ARTHUR, J. C. in collaboration with F. D. KERN, C. R. ORTON, F. D. FROMME, H. S. JACKSON, E. B. MAINS, and G. R. BISBY. The plant rusts (Uredinales). 446 pp. (New York and London). 1929.
2. BESSEY, E. A. A text-book of mycology. 495 pp. (Philadelphia). 1935.
3. CLINTON, G. P., and F. A. MCCORMICK. Infection experiments of *Pinus strobus* with *Cronartium ribicola*. Conn. Agr. Exp. Stat. Bull. 214: 428–459. 1919.
4. HIRT, RAY R. The progress of blister rust in planted northern white pine. Jour. For. 34: 506–511. 1936.
5. ————. Relation of stomata to infection of *Pinus strobus* by *Cronartium ribicola*. Phytopath. 28: 180–190. 1938.
6. LACHMUND, H. G. Method of determining age of blister rust infection on western white pine. Jour. Agr. Res. [U. S.] 46: 675–693. 1933.
7. ————. Resistance of the current season's shoots of *Pinus monticola* to infection by *Cronartium ribicola*. Phytopath. 23: 917–922. 1933.
8. MIELKE, J. L., T. W. CHILDS, and H. G. LACHMUND. Susceptibility to *Cronartium ribicola* of the four principal *Ribes* species found within the commercial range of *Pinus monticola*. Jour. Agr. Res. [U. S.] 55: 317–346. 1937.
9. RICHARDS, R. L. Susceptibility of different aged pine needles to blister rust and relation between the number of infections on pines and the persistence of their needles. Supp. Blister Rust News 11: 241–252. 1927.
10. SNELL, W. H. Blister rust in the Adirondacks. Jour. For. 26: 472–486. 1928.
11. ————. The relation of the age of needles on *Pinus strobus* to infection by *Cronartium ribicola*. Phytopath. 26: 1074–1080. 1936.
12. ————, and A. RATHBUN-GRAVATT. Inoculation of *Pinus strobus* trees with sporidia of *Cronartium ribicola*. Phytopath. 15: 584–590. 1925.
13. SPAULDING, P. Viability of telia of *Cronartium ribicola* in early winter. Phytopath. 12: 221–224. 1922.
14. ————. A partial explanation of the relative susceptibility of the white pines to white pine blister rust (*Cronartium ribicola*, Fischer). Phytopath. 15: 591–597. 1925.
15. YORK, H. H., and W. H. SNELL. Experiments in the infection of *Pinus strobus* with *Cronartium ribicola*. Phytopath. 12: 148–150. 1922.
16. ————, ————, and A. RATHBUN-GRAVATT. The results of inoculating *Pinus strobus* with the sporidia of *Cronartium ribicola*. Jour. Agr. Res. [U. S.] 34: 497–510. 1927.

STUDIES ON POLYPORUS ABIETINUS

I. THE ENZYME-PRODUCING ABILITY OF THE FUNGUS[1]

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(Accepted for publication July 12, 1938)

INTRODUCTION

The activity of wood-destroying fungi is conditioned by their ability to form a variety of enzymes. This fact has been demonstrated by several investigators, among whom are Schmitz and Zeller (16), working with *Polyporus lucidus* (Leys.) Fries (*Ganoderma lucidum* (Leys.) Karst.), *Armillaria mellea* (Vahl.) Fr., and *Daedalea confragosa* (Bolt.) Fr., and Zeller (19) working with *Lenzites saepiaria* (Wulf.) Fr. The object of the present study, therefore, was to determine the kinds of enzymes produced by the coniferous sapwood-destroying fungus *Polyporus abietinus* (Dicks.) Fr.; to compare the enzyme-producing ability of this fungus with that of

[1] A portion of a thesis submitted to the Graduate Faculty of Duke University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] The author herewith gratefully acknowledges his indebtedness to Dr. Frederick A. Wolf, under whose guidance this work was conducted.

other xylophilous fungi; and to compare 2 methods of demonstrating the production of enzymes.

METHODS

The methods for determination of enzymes used in this study are: (a) the *in vitro* method, in which an extraction of the fungal hyphae is added to solutions of various substrates. After incubation, tests are made for the presence of appropriate end products; and (b) the *in vivo* method, developed by Crabill and Reed (5), in which the fungus is cultured on a medium containing a single organic nutritive material. Either the growth of the organism or some change shown by the indicator is regarded as proof that the fungus produces the enzyme necessary for utilization of the organic substance.

In the beginning of the study a vigorous polysporic culture of the fungus was isolated by inverting a sporophore over malt agar. Transfers from this original culture were used throughout the study.

For the *in vitro* tests the fungus was grown for 3 months, in flasks, on autoclaved loblolly pine sawdust. The contents of the flasks were then triturated with sterile sand, and allowed to extract overnight in cold water. After filtration, the enzymes were precipitated from this solution with 2 volumes of 95 per cent alcohol. This preparation was filtered through a Buchner funnel, and the precipitate on 3 sq. cm. of filter paper constituted the enzymic extract from 20 g. of material. Strips, 3 sq. cm. in area, of this filter paper with the adhering enzymic extract were placed in test tubes with 2.5 per cent solutions of the substances listed in table 1, and incubated at 30° C. for the indicated periods. Toluol was used as an antiseptic, and, unless otherwise indicated, the usual methods for determination of end products were used.

In a few cases the methods of testing for enzymic activity were different from standard methods and deserve further mention. In the tests for lipase the substrate was alkalized with sodium hydroxide, and brom-thymol-blue was added as an indicator. Under these conditions the activity of lipase would result in the formation of acids, changing the indicator from blue to yellow. The same procedure was followed in testing for peptic activity. In testing for tryptic activity the blood fibrin was stained with Congo red, and fixed with hot water. Release of the dye is, in this case, an indication of the action of trypsin. The tryptophane test was applied in testing for the activity of erepsin. In tests for urease and asparaginase, rosolic acid was added to the substrates, and, if reddening of the solutions occurred, it indicated the production of ammonium by the activity of these enzymes. In the tests for maltase, lactase, and tanninase, the Barfoed method of testing for monosaccharides in the presence of reducing disaccharides was used in preference to the complex Schaffer method. In the test for activity of tanninase the solution was precipitated with albumin, and neutralized with excess sodium hydroxide before testing for monosaccharides.

Two types of controls were set up in this series of tests: (a) autoclaved strips of the filter paper with the adhering precipitated enzymes were placed in contact with the substrates, and (b) strips of filter paper on which no precipitated enzymes were suspended in similar substrates. The controls were incubated along with the tests.

The filter paper itself constituted the substrate in the tests for cellulase and cellobiase. In this case the filter-paper strips, with the adhering precipitated enzymes, were suspended in distilled water. A series of tubes was prepared sufficient to make tests with Fehling's solution at intervals of 12 hours. As a result it was determined that no appreciable hydrolysis of the cellulose had occurred prior to 12 days. The extended period required for the production of reducing sugars by the hydrolysis of cellulose makes it posssible to avoid misinterpreting the results in all other tests in which cases reducing sugars appeared rather promptly.

In the application of the *in vivo* tests, 2.5 per cent of each of the materials listed in table 1 was added to a mineral nutrient stock medium. This stock medium was composed of the following:

$MgSO_4$, 0.5 grams; KCl, 0.5 grams; K_2HPO_4, 1.0 grams; $FeSO_4$, trace; Agar, 20 grams; Water, 1000 cc.

Plates were poured with these stock media plus the various materials and were inoculated with *Polyporus abietinus*.

In tests in which negative results were obtained with this stock medium, the tests were repeated with the same medium enriched by the addition of 2.9 g. of $NaNO_3$. Tests in which both media were used are indicated with an asterisk.

Only the *in vitro* method was used in testing for oxidizing enzymes.

RESULTS

A summary of the results of these tests to determine the enzyme-producing ability of *Polyporus abietinus* is presented in table 1.

DISCUSSION

In this study positive evidence was obtained for the formation of 19 kinds of enzymes by *Polyporus abietinus*. Negative results were obtained in the tests for the hydrolytic enzyme lactase, and for the oxidizing enzyme tyrosinase. With the exception of Zeller's study (19), this list of enzymes is more extensive than lists previously reported for wood-destroying fungi. Moreover, the kinds of enzymes produced by *P. abietinus* are essentially the same as by *Lenzites saepiaria* listed by Zeller. Since both *P. abietinus* and *L. saepiaria* are potent destroyers of coniferous sapwood, it might be expected that they would be able to form similar enzymes.

An appreciation of the enzyme-forming ability of *Polyporus abietinus* can best be gained by comparison with the published results of similar studies involving other wood-inhabiting species. These comparisons can be presented most succinctly in tabular form in table 2.

TABLE 1.—*Enzymes produced by Polyporus abietinus, grown on a mineral nutrient stock medium of* $MgSO_4$, KCl, K_2HPO_4, $FeSO_4$, *agar, and* H_2O

Enzyme tested for	Substrate	Incubation *in vitro*	Period, days *in vivo*	Results *In vitro*	Results *In vivo*
Sucrase	Sucrose	3	7	+	+
	Raffinose	3	9	+	–
Maltase	Maltose	14		+	
	Amygdalin	7	26	+	–
Emulsin	Raffinose	7	26	+	–
	Salicin	7		+	
Cellulase (and cellobiase)	Filter paper cellulose[a]	14	9	+	+
Lactase	Lactose[a]	14	7	–	–
Amylase	Corn starch	3	7	+	+
Inulase	Inulin[a]	3	7	+	+
Pectinase	Pectin		9	+	–
Lipase	Methyl acetate	5	14	+	+
Pepsin	Blood fibrin	5	26	+	–
Trypsin	Blood fibrin	14	26	+	–
Erepsin	Peptone	14	26	+	–
Urease	Urea	4			+
Asparaginase	Asparagin[a]	4	7	+	+
Tanninase	Tannic acid	5	7	+[b]	+
Ligninase	Isolated lignin[a]		26		+
Oxygenases	10% alcoholic gum guaiac	½		+	
Laccase	Tannic acid	½		+	
Tyrosinase	Tyrosine	10		–[c]	
Peroxidase	Gum guaiac and H_2O_2	1 hour		+	
Catalase	H_2O_2	½ hour		+	

[a] Test repeated on mineral nutrient stock medium enriched by addition of 2.9 g. of $NaNO_3$.

[b] + indicates positive evidence.

[c] – indicates negative evidence.

Tests were made by Zeller (19) with *Lenzites saepiaria,* and in the present study with *Polyporus abietinus* for a larger number of enzymes than is the case with any of the other fungi. Certain of them, if appropriate tests were made, might be found to form other kinds of enzymes. The assembled data do indicate, nevertheless, that there is considerable variation in enzyme-producing ability among xylophilous fungi, particularly in reference to sucroclastic and proteolytic enzymes. Table 2 further reveals that *P. abietinus, L. saepiaria,* and *Daedalea confragosa* are quite diversified in their enzyme-producing ability. They secrete several enzymes not formed by species of heartwood-destroying fungi, such as *Polyporus squamosus, P. sulfureus,* and *Echinodontium tinctorium,* nor by the highly specialized cambium-attacking fungus *Armillaria mellea.*

The evidence obtained herein for the production of both cellulase and ligninase by *Polyporus abietinus* corroborates the results of Campbell (4) who found that coniferous sapwood decayed by this fungus is lower in both cellulose and lignin than is undecayed wood.

With the exception of inulase, all of the hydrolytic enzymes demonstrated for *Polyporus abietinus* are necessary for the complete utilization of some

TABLE 2.—*Kinds of enzymes produced by various wood-inhabiting species of Basidiomycetes in comparison with those produced by Polyporus abietinus*

Investigator	Organism	Enzymes																				
		Emulsin	Cellulase	Ligninase	Amylase	Sucrase	Maltase	Lactase	Inulase	Pectinase	Tanninase	Pepsin	Trypsin	Erepsin	Urease	Asparaginase	Lipase	Oxygenase	Peroxidase	Tyrosinase	Laccase	Catalase
The writer	Polyporus abietinus (Dicks.) Fr.	+[a]	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+
Schmitz (14)	Polyporus volvatus Pk.	+	+		+	+	+	+	+		+	−		−	−		+					+
Buller (3)	Polyporus squamosus Huds.	+	−		+	−	−					+					+			+	+	
Bayliss (1)	Polyporus versicolor (L.) Fr.	−[b]	+	+	+	+					+	+		+			−			+		
MacDonald (9)	Polyporus betulinus (Bull.) Fr.	+			+	−	−				+	+		−			+			+	+	
Nutman (11)	Polyporus hispidus (Bull.) Fr.	+	+	+		+												+				+
Bourquelot and Herissey (2)	Polyporus sulfureus Bull.	+	+		−	−	+		−		+											
Schmitz and Zeller (16)	Polyporus lucidus (Leys.) Fr.	+	+			+	+	+	+		+		+	+	+		+	+				
Schmitz (14)	Fomes igniarius (L.) Gill.	+	+		+	+	+	+	+		−	+			+		+					+
Schmitz (15)	Fomes pinicola Fr.		+		+	+	+	+	+		+				−		+					+
Montgomery (10)	Fomes fraxineus (Bull.) Cooke	+	−		+	+				+							+	+	+			+
Schmitz and Zeller (16)	Armillaria mellea (Vahl) Fr.				+	+	−	−	+								−	+	+			+
Schmitz and Zeller (16)	Daedalea confragosa (Bolt.) Fr.	+			+	+	+	+	+		+		+	+	+		+	+				
Schmitz (13)	Echinodontium tinctorium E. & E.		+		+	+	+	+	+			−		−	+		+					+
Zeller (19)	Lenzites saepiaria (Wulf.) Fr.	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+			+
Findlay (6)	Paxillus panuoides Fr.	+	+		+																	
Putterill (12)	Schizophyllum commune Fr.	+		−	+	+											+					+
Venkatarayan (17)	Ganoderma lucidum (Leys.) Karst.	−				+	−					+									+	−

[a] + indicates positive evidence.
[b] − indicates negative evidence.

material found in coniferous sapwoods. In spite of the fact that certain of these materials (glucosides, sucrose, and proteins) are found in minute quantities, it is shown that the fungus is able to utilize them. Consequently, it is evident that materials stored in the parenchyma cells of the sapwood are of importance in the nutrition of this fungus. Wilson (18) has shown that the amount of food stored in parenchyma cells of wood influences the liability of attack of wood by insects and sap-staining fungi. In view of the present evidence, it is equally logical to assume that the amount of stored food likewise influences the rate of growth of sapwood-destroying fungi.

Of the 21 enzymes tested for in this study, 7 were demonstrated by the *in vitro* method, which could not be shown by the *in vivo*. Recently, however, the determination of plant enzymes *in vitro* has been subjected to considerable criticism. The more valid objections to the *in vitro* method, as discussed by Kertesz (7) are: (a) the possibility that the treatment during extraction diminishes or inhibits the activity of enzymes; (b) the possibility that, when extracted from the cells, the proteolytic enzymes may digest some of the other enzymes; (c) the possibility that all enzymes may not be soluble in the solvent used, and, hence, may not be extractable; (d) the difficulty of establishing that there has been no bacterial activity during the extraction or in the substrate.

It is obvious that none of these objections may be leveled at the *in vivo* method are herein employed. It would seem that an easily-cultured saprophytic fungus affords an excellent opportunity for demonstrating enzymatic activity in vigorously growing material. In the majority of tests in which the results from the 2 methods are contradictory, it is evident that the single organic material used, even though hydrolyzed, would not support growth and activity of the fungus. The employment of the *in vivo* method, with appropriate modifications, as a substitute for the *in vitro* method for demonstrating enzymatic activity is, nevertheless, highly desirable in future investigations with microorganisms.

SUMMARY

The coniferous-sapwood-destroying fungus *Polyporus abietinus* is herein shown to form 15 of the more common hydrolytic enzymes, namely, emulsin, cellulase, ligninase, amylase, sucrase, maltase, inulase, pectinase, tanninase, pepsin, trypsin, erepsin, urease, asparaginase, and lipase; and is shown to form 4 of the more common oxidizing enzymes, namely, oxygenase, peroxidase, laccase, and catalase.

Polyporus abietinus produces more kinds of enzymes than have been previously reported for other wood-destroying fungi, with the exception of *Lenzites saepiaria*.

The variety of hydrolytic enzymes produced by *Polyporus abietinus* indicates that materials stored in the wood parenchyma are of considerable importance in the nutrition of the organism.

A comparison of results obtained by the *in vitro* and *in vivo* methods indi-

cates that the former more accurately demonstrates total number of enzymes that a given microorganism is capable of producing.

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LITERATURE CITED

1. BAYLISS, J. S. The biology of *Polistictus versicolor* (Fr.). Jour. Econ. Biol. **3**: 1–24. 1908.
2. BOURQUELOT, E. and H. HERISSEY. Les ferments solubles du *Polyporus sulfureus* Bull. Bull. Soc. Mycol. France **11**: 235–239. 1895.
3. BULLER, A. H. R. The enzymes of *Polyporus squamosus* Huds. Ann. Bot. **20**: 49–59. 1906.
4. CAMPBELL, W. G. A chemical approach to the study of wood preservation. Forestry (Gt. Brit.) **6**: 82–89. 1932.
5. CRABILL, C. H. and H. S. REED. Convenient methods for demonstrating the biochemical activity of microorganisms with special reference to the production and activity of enzymes. Biochem. Bull. **4**: 30–44. 1915.
6. FINDLAY, W. P. K. A study of *Paxillus panuoides* Fr. and its effect upon wood. Ann. Appl. Biol. **19**: 331–350. 1932.
7. KERTESZ, Z. I. On the determination of plant enzymes. Plant Physiol. **12**: 845–851. 1937.
8. LANPHERE, W. M. Enzymes of the rhizomorphs of *Armillaria mellea*. Phytopath. **24**: 1244–1249. 1934.
9. MACDONALD, J. A. A study of *Polyporus betulinus* (Bull.) Fr. Ann. Appl. Biol. **24**: 289–310. 1937.
10. MONTGOMERY, H. B. S. A study of *Fomes fraxineus* and its effects on ashwood. Ann. Appl. Biol. **23**: 465–486. 1936.
11. NUTMAN, F. J. Studies of wood destroying fungi. I. *Polyporus hispidus* Fr. Ann. Appl. Biol. **16**: 40–64. 1929.
12. PUTTERILL, V. A. The biology of *Schizophyllum commune* Fr., with special reference to its parasitism. Union South Africa, Sci. Bull. Dept. Agr. S. Africa. **25**: 35 pp. 1922.
13. SCHMITZ, H. Enzyme action in *Echinodontium tinctorium* E. and E. Jour. Gen. Physiol. **2**: 613–616. 1920.
14. ———. Enzyme action in *Polyporus volvatus* Pk., and *Fomes igniarius* (L.) Gill. Jour. Gen. Physiol. **3**: 795–800. 1921.
15. ———. Studies in wood decay. V. Physiological specialization in *Fomes pinicola* Fr. Amer. Jour. Bot. **12**: 163–177. 1925.
16. ———, and S. M. ZELLER. Studies in the physiology of the fungi. IX. Enzyme action in *Armillaria mellea* Vahl., *Daedalea confragosa* (Bolt.) Fr., and *Polyporus lucidus* (Leys.) Fr. Ann. Mo. Bot. Gard. **6**: 193–200. 1919.
17. VENKATARAYAN, S. V. The biology of *Ganoderma lucidum* on areca and cocoanut palms. Phytopath. **26**: 153–175. 1936.
18. WILSON, S. E. Changes in the cell contents of wood and their relationship to the respiration of wood and its resistance to Lyctus attack and to fungal invasion. Ann. Appl. Biol. **20**: 661–690. 1933.
19. ZELLER, S. M. Studies in the Physiology of the fungi. II. *Lenzites saepiaria* Fr. with special reference to enzyme activity. Ann. Mo. Bot. Gard. **3**: 439–512. 1916.

HOST-PARASITE RELATIONS OF SCLEROSPORA GRAMINICOLA ON SPECIES OF SETARIA[1]

E. S. McDonough[2]

(Accepted for publication July 15, 1938)

Infection of *Setaria italica* (L.) Beauv., *Panicum miliaceum* L., *Euchlaena mexicana* Schrad., and *Zea mays* L. with germinating oospores of *Sclerospora graminicola* (Sacc.) Schroet. was first reported by Melhus and Van Haltern (3) in 1925. Later, Melhus, Van Haltern, and Bliss (4) reported infecting species in 5 genera of the Gramineae with *S. graminicola*, and that infection of the seedlings took place between germination and emergence. Since this early work, numerous investigators have demonstrated the infectious nature of *Sclerospora* oospores on a wide range of host plants.

Hiura (1) placed germinating oospores directly on the young plant parts, and reported that infection took place through either the coleoptile, the root, or the mesocotyl. Oospores were reported capable of causing infection within 4 days after they had been sown. The hyphae were reported to have penetrated the epidermis of the host. Such a penetration, however, was not illustrated nor was it described in detail.

The present study on the host relationship of *Sclerospora graminicola* on *Setaria viridis* and *S. italica* was initiated in 1935. The entrance of the infection hypha emanating from the oospore into the host and some observations on the subsequent development of the parasite were reported in 1937 (2). In the investigation here reported an attempt was made to study the growth of the mycelium initiated by the oospore until a systemic infection was established, the tissues of the host involved, and the general relations of the fungus to its host.

METHODS AND MATERIALS

Shredded leaves of *Setaria viridis* plants infected with *Sclerospora* were collected near Ames, Iowa, in October of 1935, near Waukesha, Wisconsin, in March, 1936, and near Milwaukee, Wisconsin, in October, 1936. Care was taken to select leaves containing oospores that had turned a reddish brown. In all instances the plants had not lodged and the leaves were thoroughly dry. Preliminary examinations of oospore material indicated that the oospores from different localities and even from parts of the same plant varied widely in the percentage of germination. For this reason only material showing the most uniformly high percentage of germination was used in infection experiments.

[1] The results reported here were obtained at Iowa State College and at Marquette University. The original report was included in a thesis submitted to the faculty of the Graduate College, Iowa State College, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] The author wishes to express his appreciation to Dr. J. E. Sass under whose direction the investigation was carried out and to Dr. I. E. Melhus who suggested the problem and who gave invaluable advice during the course of the work.

The following method was used to expose seedlings to infection: The seed was placed on moist cotton in a Petri dish, the cover of which also had been lined with moist cotton. Such seed was then covered with shredded leaf material containing the oospores and allowed to stand at room temperature. One hundred hours after the seed had been sown, the Petri dishes were transferred to bell jars near a window, and the lids removed, care being taken to keep the cotton moist. Small beakers of water were placed under the bell jars with the Petri dishes. Conidia usually were observed on susceptible plants 5 days after the seed had been covered with the oospores. Preliminary observations of infection were made with material stained and fixed in aceto-carmine.

Artificially infected material that was to be sectioned in paraffin was killed in standard chromo-acetic and in a solution containing 75 ml. of 1 per cent acetic acid, 20 ml. of 1 per cent chromic acid and 5 ml. of 37 per cent formaldehyde. By careful dissection under a binocular microscope it was possible to obtain very young, whole seedlings for preservation. The fertile lemma was removed from the seed with a dissecting needle. As soon as the lemma had been removed, the seedling was carefully cut from the fruit and dropped into the fixing fluid. Very little shrinkage of material was observed, probably because of the rapidity with which the dissection was made and also because of the more or less noncutinized condition of the seedling.

EXPERIMENTAL RESULTS

In March, 1936, seeds of *Setaria italica* (Siberian variety) were exposed to infection. As a rule the coleorhiza was the first part of the seedling to emerge, although under exceptional conditions the coleoptile was first. As soon as the coleorhiza had emerged, the young seedlings from one lot were dissected out of the fruit and preserved. Twenty-four hours later the roots of the remaining plants averaged 4 mm. in length, and the seedlings from another dish were dissected out and fixed. Two more dissections were made at intervals of 24 hours. Fourteen days after the seeds had been sown the seedlings were cut off just above the place where they entered the cotton.

In June, 1936, the experiment described above was repeated. However, at the end of 48 hours, because of the higher temperature at this time, some of the seeds had produced seedlings with roots 4 mm. long. Such seedlings were dissected out of the fruit and mounted in aceto-carmine. In June, 1937, seeds of *Setaria viridis* were exposed to infection and the seedlings fixed as described above for *S. italica*.

Observations made on the material prepared by the aceto-carmine method showed that the fungus entered the root or coleorhiza by penetrating directly through the epidermal cells. It was apparent from such observations that the entrance of the germ tube could take place at an early period in its development. Figure 1, A, was made of an oospore that had germinated in contact with the coleorhiza. The growth of the hypha after it had entered the tissue of the host was followed for some time in the material stained with

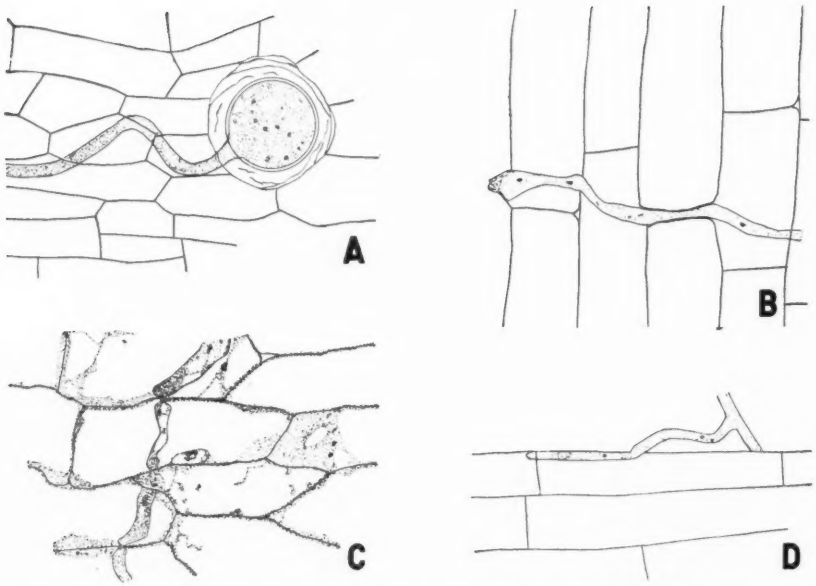

FIG. 1. A. An oospore whose germ tube had penetrated into the coleorhiza of a 24-hour-old *Setaria italica* seedling. ×150. B. Hypha entering root of *S. italica*. ×150. C. Entrance of an infection hypha into the epidermal cell of an *S. italica* root. ×150. D. Intracellular growth of Sclerospora hypha in the coleorhiza of *S. viridis*. ×225.

aceto-carmine, but, because of the thickness of such preparations, it was found impossible to continue accurately the observations of the history of the infection hypha in older seedlings. Therefore, such observations were made on plant material imbedded in paraffin (Fig. 2).

The results of the present investigation indicated that the germ tube had penetrated directly into the seedling through the epidermal cells. Occasionally, as is indicated in figure 1, D, the germ tube was found to branch before it had entered the root of the host. The investigations did not disclose the extent of the growth of the germ tube that occurred between germination and the time the infection. It would seem, however, that this growth was rather limited.

Germ tubes were observed to have entered the coleorhiza, the root, the coleoptile, and the mesocotyl. The larger number of infections was, however, observed to have taken place through the epidermis of the root and the coleorhiza. Germ tube penetration into the very young embryonic region of the root or into the root cap was observed to have taken place only rarely. It is likely that the present method of inoculation did not favor the infection of the tip of the root, or the penetration of the germ tube into the regions of the seedling that, normally, would grow above ground. No infection was observed to take place through root hairs.

After the infection hypha had entered the epidermal cell it was found to penetrate directly through the host cell or to branch within the cell. Further development of the hypha or a branch from the original infection

thread was found to be intracellular until several of the host cells had been penetrated. Eventually, the parasite became intercellular. However, even after the mycelium had penetrated for some distance into the root, hyphae were found, at times, to have passed through the cell walls (Figs. 1, C, and 2, A). Haustoria were found on intercellular mycelium shortly after infection had taken place.

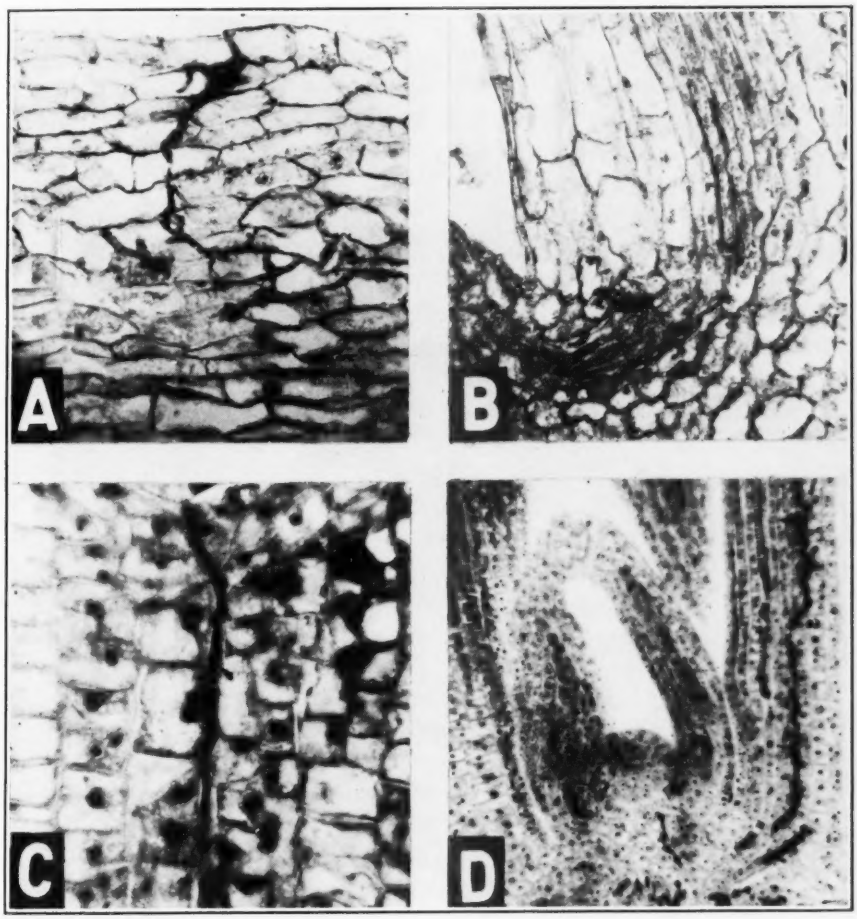

FIG. 2. A. Entrance of infection hypha into the coleorhiza. B. Mycelium in cotyledonary node and in the base of the mesocotyl. C. Hypha directly below the plumule. A haustorium may be seen. D. Mycelium in the embryonic region. A, B, D: ×207. C, ×480. Photomicrographs by F. A. Bautsch, S.J.

In certain instances, after the germ tube had penetrated into a cell, it was observed to have swollen considerably on both sides of the wall at the point of entrance. An interesting example of this is illustrated in figure 1, B, in which it may be seen that the infection hypha, soon after having entered into the epidermal cell of the host, has increased greatly in size at a point next to the outer wall of the host cell. A piece of the wall has been pushed outward under the pressure developed by the hypha.

Sections of plants that had been fixed when the coleorhiza was just emerging from the seed were found, in some instances, to have already become infected. In such cases the infection hyphae of the fungus could be seen to have entered the coleorhiza. Two of 10 seedlings so sectioned at this stage were found infected. In no case was the mycelium observed in sections of embryos of ungerminated seeds that had been exposed to infection. No infection was seen in sections made of the control seedlings nor were conidia observed at any time on any of the control cultures.

Sections of seedlings made 24 hours after they had started to emerge from the seed showed the fungus present in 5 of the 7 seedlings examined. In one instance, the growth of the mycelium of *Sclerospora* was limited to the root; in 2, to the coleorhiza; and in 3, it was found in all parts of the young seedling except the coleoptile.

Roots of seedlings 24 hours old often showed an extensive growth of the fungus. Although it was observed that the fungus spread through the root, the actual amount of mycelium found in the roots was not great, even in plants with mycelium in the embryonic stem. The coleorhiza at times contained a widespread mycelium at an early stage. Some preparations indicated, without doubt, that the initial infection had taken place through the coleorhiza. It was not uncommon, however, to find the root, as well as the coleorhiza, infected at several points. Because of the intimate connection of the coleorhiza with the rest of the seedling, and especially because of the easy access afforded by the cotyledonary node to the parts of the plant aboveground, it seemed that the typical infection point was the coleorhiza.

The cotyledonary (scutellary node) was observed to contain the hyphae of *Sclerospora* in seedlings 24 hours old. This mycelium was found in some instances continuous with that of the root and at other times with that of the coleorhiza.

The cotyledon (scutellum) also became infected by the pathogen. Mycelium was observed ramifying through the cotyledon, but not densely filling it. The hyphae had penetrated as far as the epithelial layer.

It was observed in some cases that the mesocotyl had been entered directly by the pathogen. Under the conditions of these experiments the spread of the pathogen into the mesocotyl was found to occur from the cotyledonary node, which, in turn, had become infected by mycelium from the coleorhiza or the root. It was possible to trace the mycelium in some seedlings from the initial infection in either the root or the coleorhiza to the mesocotyl (Fig. 2, A, B). Once having entered the mesocotyl, the growth of the fungus was directed almost entirely toward the embryonic region of the stem. The path of the mycelium was through either the cortex of the mesocotyl or through the stele. As is shown in figure 2, C, D, the hyphae entered directly into the meristematic region. From this region the fungus infected the young leaves and branches as they were produced (Fig. 2, D).

In 14-day-old plants that were sectioned were found hyphae bearing conidiophores. The hyphae found in the nodes and internodes of such

plants did not stain heavily in the region of the meristem. Such hyphae were apparently inactive at that time.

Plants infected with *Sclerospora* showed a darkening of the primary root, accompanied in some cases by necrosis. Upon examination such roots were found invaded by the mycelium of *Sclerospora* and also that of other fungi. Spores of *Helminthosporium* and *Fusarium* were found in dishes containing seedlings with necrotic roots, and it can be assumed that the septate mycelium observed was that of one of these fungi. Such roots often were observed to contain much mycelium and to rot away completely. On the other hand, apparently healthy roots showed on examination the presence of the mycelium of *Sclerospora*. The roots of young plants that had produced conidia were examined macroscopically and many plants were found with normal roots.

Stunting, caused by a shortening of the mesocotyl and of the internodes, was one of the most noticeable effects of the pathogen on the early development of the host seedlings. Such a dwarfing was often noticeable even in plants only a few days old. However, after 14 days of growth, some seedlings that produced conidia were observed to be as tall as the checks.

Longitudinal sections of dwarfed seedlings, 14 days old, showed marked areas at the top of the mesocotyl, and in the internodes of the plant. Such areas were often located in the meristematic tissues of these regions. At earlier stages it was observed that the mycelium in the plumule caused a local necrosis, apparently brought about by substances directly or indirectly produced by the fungus, since cells not in contact with the pathogen were found to be necrotic. Because of these observations it was thought that the dwarfing might have resulted partly from a necrosis of meristem cells. Further work on this phase of the problem is contemplated.

SUMMARY

Infection of *Setaria italica* and *S. viridis* seedlings by *Sclerospora graminicola* was found to take place through either the root, the coleorhiza, the mesocotyl, or the coleoptile. The germ tube of the organism, or a branch from it, was observed to pass directly through the epidermal cells.

Seedlings were observed infected when the coleorhiza was just emerging from the seed. Since the coleorhiza is usually the first part of the seedling to emerge, the natural entrance of the fungus is considered to be most often through this organ.

The pathogen was observed to have become systemic before the mesocotyl had elongated to any extent and before the coleoptile had penetrated far from the seed.

The early growth of the fungus in the plant was found to be intracellular, but an intercellular mycelium with haustoria was formed after the infection hypha had penetrated a few cells of the host.

After the fungus had entered the plant, growth was directed toward the embryonic stem tip. From this region the mycelium entered the young leaves of the plant as they developed.

LITERATURE CITED

1. HIURA, M. Mycological and pathological studies on the downy mildew of Italian millet. Jour. Fac. Agr. Hokkaido Imp. Univ. Sapporo 36: 121–283. 1935.
2. MCDONOUGH, E. S. Primary infection of *Setaria italica* (L.) Beauv. by *Sclerospora graminicola* (Sacc.) Schroeter. Phytopath. 27: 311–313. 1937.
3. MELHUS, I. E., and VAN HALTERN, F. Sclerospora on corn in America. Phytopath. 15: 720–721. 1925.
4. ———, ———, and BLISS, D. E. A study of *Sclerospora graminicola* (Sacc.) Schroet., on *Setaria viridis* (L.) Beauv. and *Zea mays* L. Iowa Agr. Exp. Stat. Res. Bull. 111: 297–338. 1928.

NAUCORIA ON SMALL GRAINS IN ILLINOIS

G. H. BOEWE

(Accepted for publication May 9, 1938)

In the summer of 1935, the writer found a small gill fungus of the genus *Naucoria* growing on wheat plants in Massac County, Illinois. Only a small area in the field, a hillside with west exposure, was found infested; and relatively few plants in the area were attacked. When this collection was made, May 16, most of the plants were headed out. In 1936, the same field was in wheat and again the fungus was found on this host in the same area in the field, but the intensity of attack was less, probably due to the dry weather of that year. Later in the summer of 1935, the same fungus was found on barley and rye in widely separated counties of the State.

Many species of *Naucoria* grow on dead plants and on soil, but very few have been reported as definitely attacking living plants. Indeed, relatively few agarics grow on living herbaceous plants and induce disease. According to Cook (4), however, a toadstool, thought to be parasitic, was found growing on sugar cane in Java, which Wakker described in 1895 as *Marasmius sacchari*. Since then, its parasitic habit has been definitely proved and its occurrence has been established in many places in warmer regions. Other species of *Marasmius* have been reported on various hosts by several workers. Cobb (2) records the occurrence of *Marasmius* on sugar cane in Hawaii, and Fulton (6) and Edgerton (5) state that sugar cane in Louisiana is attacked. According to Cook (4), "The geographical distribution of *M. sacchari* and related species may be summarized as follows,—*M. sacchari* has been reported from Java, India, Australia, Formosa, Hawaii, Puerto Rico, Jamaica, Lesser Antilles, British Guiana and South Africa; *M. plicatus* from Java, Philippines and United States; *M. stenophyllus* from Santo Domingo and Lesser Antilles; *Marasmius* spp. from Fiji, Central America and Brazil; and *Hypochnus sacchari* from Cuba and Jamaica." Cook (3) reports species of *Marasmius* causing disease on sugar cane, tea, cocoa, nutmeg, and banana. In 1922, Young (9) collected a *Marasmius* on wheat in Illinois and later described it as *M. tritici*. It was found on oats, rye, barley, *Agropyron repens* (L.) Beauv., and an unidentified grass. Mains (7) reports finding *Marasmius instititious* Fries on several clumps of *Festuca capillata* Lam. in Indiana.

In 1937, the writer (1) reported the finding in 1935 of his *Naucoria* on wheat, rye, and barley in Illinois, and, in 1938, Sprague (8) reported observing *Pholiota durata* (Bolt.) Fries, *P. praecox* (Pers.) Fries, *Naucoria* spp., and a number of small, delicate, undetermined agarics attacking or associated with crowns of oats, barley, and wheat in western Oregon.

A few species of *Naucoria* are listed in Saccardo's Sylloge Fungorum as occurring on living plants, namely, *N. musarum* Pat. and Demange on stems of mosses, *N. paludosella* Atk. on sphagnum and other mosses, and *N. pityrodes* Briganti on "semi-living" culms of *Agrostis vulgaris* With. Several other species are listed as occurring on stems of various monocotyledons, but it is not clear whether the host was living or dead. One, *N. graminicola* Nees, is reported on stalks and culms of grasses, in Europe.

The description of none of these species fits the fungus found by the writer on wheat, rye, and barley.

FIG. 1. *Naucoria* fruiting on tillers of living wheat (2 at right) and barley (left), collected in Illinois in 1935.

Most of the sporophores were attached to dead tillers (Fig. 1), but some were attached by white rhizomorphs to roots that were apparently living and functioning. By teasing apart affected tillers and the portion of the crown from which they arose, a whitish cast, due to the fungus mycelium, was observable. Examining microscopically hand sections of affected root tissues, the mycelium of the fungus was found beyond the point of attachment of the rhizomorph. The fact that this toadstool generally was found

fruiting on dead tillers and that its mycelium ramifies through the crown and roots to which it is attached, suggests that it is at least mildly parasitic, an assumption that is supported by the fact that unattacked tillers grow to nearly normal height. In rye and barley the sporophores were attached more often to crowns of the plants than to dead tillers.

The sporophore of this fungus has no ring, the spores are golden brown, the stipe is cartilaginous, the gills are not decurrent, and the margin of the pileus is at first incurved, characters that place it definitely in the genus *Naucoria*. Since it differs from all other species of this genus, the descriptions of which the writer has been able to find, he considers it a new species for which he gives the following diagnosis.

Naucoria cerealis, n. sp.

Pileus thin, membranous at first, hemispheric and then expanded, sometimes with depressed center and margin turned down, 1–2.2 cm. broad, glabrous, light buff, flesh whitish; lamellae adnate, broad, thin, chestnut; spores golden-brown, oval in one view but asymmetrically oval in the other, with a gelatinous apicule covering the germ pore, smooth, 9.9–16.5 × 6.9–9.9 μ, generally 12.5–13.5 × 7–8.5 μ; stipe equal, slightly bulbous at base, glabrous, light buff, stuffed, becoming hollow with age, striate in upper portion, 2–4.6 cm. long, 1–1.5 mm. thick.

Pileo tenui, membranaceo primitus, hemisphaerico dein expanso, nonnumquam cum centro depresso et margine deorsum curvato, glabro, argillaceo, carne albido, 1–2.2 cm. lato; lamellis adnatis, latis, tenuibus, castaneis; sporis aureis-brunneis, hinc ovalibus sed illinc asymmetrice ovalibus, cum apiculo gelatinoso supra poro germinationis, laevibus, 9.9–16.5 × 6.6–9.9 μ, plerumque 12.5–13.5 × 7–8.5 μ; stipite aequali, basi leniter bullate incrassato, glabro, argillaceo, farcto, in aetate cavitato, striato sursum, con pileo concolori, 2–4.6 cm. alto, 1–1.5 mm. crasso.

Fruiting on functioning roots and dead tillers of living wheat and on crowns and tillers of rye and barley.

Type specimen is Accession No. 25602 in the Mycological Collection of the Illinois State Natural History Survey, on wheat, Massac County, Illinois, May 16, 1935. Other collections are: No. 25603 on *Hordeum sativum* Jessen., Cora, Jackson County, June 5, 1935; No. 25413 on *Secale cereale* L. near Chadwick, Carroll County, June 22, 1935; No. 25414 on same host near Georgetown, Vermilion County, June 11, 1935; and No. 25483 on *Triticum vulgare* L., May 12, 1936, from the same field in Massac County as in the previous year.

SUMMARY

The occurrence of a *Naucoria* on wheat, barley, and rye in Illinois is reported. It is believed to be weakly parasitic, and is described as a new species, *N. cerealis*.

SECTION OF APPLIED BOTANY AND PLANT PATHOLOGY,
ILLINOIS STATE NATURAL HISTORY SURVEY,
URBANA, ILLINOIS.

LITERATURE CITED

1. BOEWE, G. H. Tiny toadstools on crop plants in Illinois. Trans. Ill. State Acad. Sci. 30 (2): 103–104. 1937.

2. COBB, N. A. Fungus maladies of the sugar cane. Hawaiian Sugar Planters Assoc. (Div. Veg. Path. and Phys.) Bull. 5 (7): 214–215. 1906.
3. COOK, M. T. The diseases of tropical plants. 317 p. MacMillan and Co., Ltd. (London). 1913.
4. ———. *Marasmius sacchari;* a parasite on sugar cane. Jour. Dept. Agr. Puerto Rico **16** (2): 213–226. 1932.
5. EDGERTON, C. W. Some sugar cane diseases. La. Agr. Exp. Stat. Bull. 120: 26 p. 1910.
6. FULTON, H. R. The root disease of sugar cane. La. Agr. Exp. Stat. Bull. 100: 21 p. 1908.
7. MAINS, E. B. Observations concerning the disease susceptibility of cereals and wild grasses. Proc. Ind. Acad. Sci. **34** (1924): 289–295. 1925.
8. SPRAGUE, RODERICK. Gill fungi associated with the roots of cereals. Phytopath. **28**: 78–79. 1938.
9. YOUNG, P. A. A Marasmius parasitic on small grains in Illinois. Phytopath. **15**: 115–118. 1925.

CHLOROTIC STREAK OF SUGAR CANE IN THE UNITED STATES

E. V. ABBOTT

(Accepted for publication June 23, 1938)

Chlorotic streak, a disease of sugar cane, until recently (1, 5) unreported in the continental United States, has been observed in Louisiana during the fall of 1937 and the spring of 1938. This disease, possibly of virus origin, is known to occur in Java, Australia, Hawaii, and Puerto Rico.

The symptoms of the disease as observed in Louisiana are identical with those described by Martin (2, 3) and Wilbrink (6). The streaks first appear as faint, narrow, elongate, chlorotic areas, with wavy, irregular margins, later becoming larger and more sharply defined, and frequently developing papery, necrotic centers (Fig. 1). In size they vary from a faint line to $\frac{3}{8}$ inch in width, and in length from a fraction of an inch to almost the entire length of the leaf. The streaks may occur on any part of the leaf blade, but do not extend to the sheath, and may appear on only one or on several leaves of an affected plant.

In order to determine whether the chlorosis was transmitted in seed cuttings, thus affording a more positive identification as chlorotic streak, cuttings of 3 affected varieties were divided into 2 lots, 1 of which was treated with hot water at 52° C. for 20 minutes, which both Wilbrink (6) in Java and Martin (3) in Hawaii found cured the disease, and the other was left untreated as a control. Single-eye pieces from each lot were then planted in steamed soil in 4-inch pots in an insect-proof greenhouse.

All of the plants arising from eyes treated with hot water came up healthy and remained so during a 6-month period of observation. Sixty-four per cent of those from nontreated eyes developed typical symptoms of chlorotic streak that persisted during the course of the experiment. The transmission of the typical symptoms in nontreated cuttings and their elimination by the hot-water treatment were considered definite proof that the disease was chlorotic streak.

When first observed in August, 1937, the disease was found on 4 unre-

leased seedling varieties (C. P. 29/99, 33/229, 33/243, and 33/253) in a test plot on one plantation. It reappeared in the ratoons of these varieties in the spring of 1938, and in plant cane, the seed for which had been cut from the affected stools in the fall of 1937. Several diseased stubbles of the C. P. 29/99 developed only 1 or 2 rather weak shoots, indicating that the disease was causing some suppression of germination.

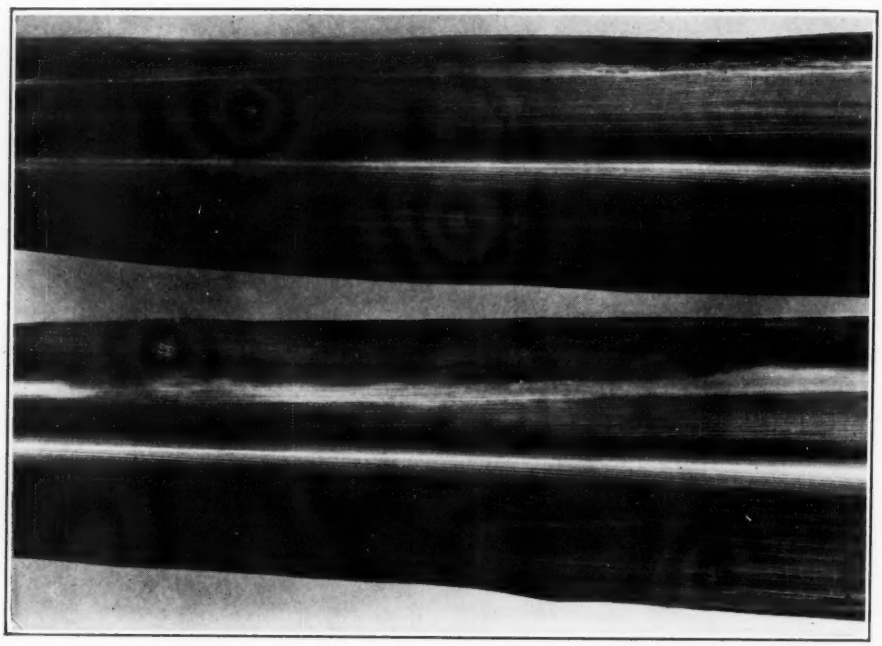

FIG. 1. Chlorotic streak on mature leaves of the sugar-cane variety C. P. 29/99. Note distinctive, wavy-margin lesions and beginning of papery, necrotic centers in the streak on the leaf at the top.

In a later survey by R. D. Rands and the writer to determine the distribution of the disease, chlorotic streak was found on additional seedling varieties on another plantation in Louisiana, and to the extent of 10 to 15 per cent in fields of the commercial variety C. P. 29/320. It is now known to occur in at least 4 parishes of that State. The disease was not found in the sugar-cane-growing sections of Florida nor in southern Georgia. A further survey of the sugar-cane district of Louisiana is now in progress.

The origin of the disease in the United States has not been determined. National quarantines Numbers 15 and 16 have prohibited importation of sugar cane from foreign countries and insular possessions since June 6, 1914. It is unlikely that this disease could have been introduced with foreign varieties imported by the United States Department of Agriculture, because of the stringent quarantine regulations. Such importations are subjected to several hot-water treatments during the course of a year or more while being propagated under observation in quarantine at Arlington Farm, Virginia,

before distribution to commercial areas. Therefore, except through possible unauthorized or smuggled importations of cuttings since 1914, the disease may have been introduced prior to the institution of the Federal quarantines, and have persisted on sugar cane or some wild-grass host without being detected, possibly on a symptomless carrier. The existence of such has not been demonstrated, and a careful inspection of grasses in the vicinity of the sugar-cane fields where the disease was found has failed to reveal symptoms more than faintly suggesting chlorotic streak.

Although no local attempts have been made to transmit the disease, either by artificial inoculation or by insects, efforts in other countries (4), involving various mechanical methods and such insects as the cane aphid and leaf hopper, the corn aphid, and the onion thrips, have given only negative results.

Of the present commercial varieties, C. P. 29/320 appears to be very susceptible to infection. Other commercial varieties with apparently equal exposure to the disease have contracted it to a much less extent. The information available at present is, however, unsufficient to permit a classification of varieties with respect to susceptibility to the disease. The hot-water treatment offers a means for starting healthy seed plots of infected susceptible canes.

DIVISION OF SUGAR PLANT INVESTIGATIONS,
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LITERATURE CITED

1. ABBOTT, E. V. Chlorotic streak. The Sugar Bull. **16** (17): 3–4. 1938.
2. MARTIN, J. P. Chlorotic streak disease of sugar cane. Hawaiian Plant. Rec. **34**: 375–378. 1930.
3. ————. Chlorotic streak disease of sugar cane. Proc. 5th Congr. Intern. Soc. Sugar Cane Techn. **5** (1935): 823–828. 1936.
4. ————. Chlorotic streak. *In* Experiment Station Report. Ann. Meeting Hawaiian Sugar Plant. Assoc. **56**: 30. 1937.
5. TAGGART, W. G., C. W. EDGERTON, E. V. ABBOTT, and J. W. INGRAM. Chlorotic streak (Committee Report). The Sugar Bull. **16** (18): 2–3. 1938.
6. WILBRINK, G. Diseases resembling leaf scald. Proc. 4th Cong. Intern. Soc. Sugar Cane Techn. Bull. 117. 8 pp. 1932.

PHYTOPATHOLOGICAL NOTES

Relations between Crown Gall and pH of the Soil.—Almost 20 years ago, Dr. M. B. Waite informed the writer that he considered crown gall to be the most serious in regions where the soil is relatively alkaline. Later, when comparisons were made between organisms isolated from various malformations on apple and other hosts the writer[1] called attention to the fact that the organism isolated from "woolly knots" or "hairy roots" of apple exhibited striking differences from the crown-gall organism, *Phytomonas tumefaciens* Smith and Town., in host specificity. A difference in their reaction in litmus milk has frequently been observed. This organism, later classified as *Phyt. rhizogenes* Riker *et al.*,[2] produces growth in media as acid as pH 4.0;[2] the crown-gall organism produces most growth at pH 7,[3] with pH limits at 9.6 to 5.2[4] or 4.4.[2] Accordingly, general observations have been made over a period of years on the prevalence of these two diseases in relation to the pH of the soil. As an example, the situation encountered during an inspection of nurseries growing peach trees in the Willamette Valley, Oregon, and in the Sacramento River Valley, California, may be cited. The soils in the Willamette Valley are relatively acid, and liming is practiced for certain crops; in the nursery in this section almost no crown gall was found. In the nurseries in the Sacramento River Valley, where the soil in general is relatively alkaline, crown gall infections were abundant.

In order to test the hypothesis that relatively alkaline soils may be a predisposing factor for crown-gall infections, 4 adjacent rows, approximately 75 feet long, were selected for planting peach seed. The soil was known to be acid. Two alternate rows were limed in the fall of 1937, and, 1 week later, all 4 rows were inoculated heavily with a water suspension of the crown-gall organism. The seedlings were dug in the fall of 1938 and composite samples

TABLE 1.—*Comparison of the amount of crown gall on peach seedlings grown in*[1] *relatively acid and in*[2] *approximately neutral soils*

Row	Treatment	Number of trees	Number of galled trees	Galled trees (per cent)	pH of soil
1	Limed	360	115	32	6.8
2	Not limed	337	12	4	5.0
3	Limed	452	147	33	6.8
4	Not limed	341	6	2	5.0
Total	Limed	812	262	32	6.8
"	Not limed	678	18	3	5.0

[1] Siegler, E. A. Studies on the etiology of apple crown gall. Jour. Agr. Res. [U.S.] **37**: 301–313. 1928.

[2] Riker, A. J., Banfield, W. M., Wright, W. H., Keitt, G. W. and Sagen, H. E. Studies on infectious hairy-root of nursery apple trees. Jour. Agr. Res. [U.S.] **41**: 507–540. 1930.

[3] Patel, M. K. Biological studies of *Pseudomonas tumefaciens* Smith and Town., and fifteen related non-pathogenic organisms. Iowa State College Jour. Sci. **3**: 271–298. 1929.

[4] Personally communicated by Agnes J. Quirk.

of soil from each of the 4 rows were taken for pH determinations. The results are given in table 1.

The results show that 32 per cent of the seedlings grown in the limed rows were galled and that 3 per cent of the trees grown in the relatively acid rows were galled. It is highly desirable to confirm these results with similar experiments. Until more data are obtained, however, it is thought that caution should be exercised before advising nurserymen to lime their soils where crown gall is an important factor. It is possible that adjustment of the pH of certain soils may likewise prove to be an aid in controlling hairy root.—E. A. SIEGLER, U. S. Horticultural Station, Beltsville, Maryland.

Spine Development on the Spores of Ustilago zeae.—Ustilago spore formation was first noted in detail by de Bary in 1853; some additional contributions, especially with regard to the cytology were made by Lutman and Seyfert. The mycelium forms jelly-like nests composed of hyphae in worm-like coils among the corn cells. At maturity, the hyphae break up into short, binucleate bodies surrounded by a thick wall of jelly. In the final stage the thick, jelly-like wall becomes brown and covered with spines.

The development of a spiny wall from a gelatinous matrix is somewhat puzzling and seems not to have been studied, perhaps because of the small size of the smut spores. A permanent stain was sought that would bring out the gelatinous layer, the developing wall with its spines, and at the same time reveal, as much as possible, the spore contents. Hutchinson's stain combination[1] of orseilline BB (Grübler) with aniline blue, used in staining freshly-cut sections of white pine for the blister-rust mycelium, was among the combinations tested. He found "mycelium stained violet to blue, suberized and lignified walls are red. Haustoria . . . stain a deep red in contrast to the bluish mycelium outside the cell wall." This combination was Strasburger's adaptation of Mangin's discovery of the value of orseilline (a dye obtained from various lichens) as a test for cellulose. The technique used for staining the sections of corn bolls infected with *Ustilago zeae* was different in a number of steps from that used by Hutchinson.

Sections from material fixed in the Allen-Bouin mixture were transferred to water and then stained in a saturated orseilline BB solution, about 1 gram of orseilline in 30 cc. of 3 per cent acetic acid, for 24 hours. 50 per cent alcohol was then run quickly over the slide to remove the excess stain, and the sections were then placed in a saturated solution (1 g. of aniline blue in 100 cc. of 3 per cent acetic acid) for 24 hours. The sections were dehydrated quickly by flushing with absolute alcohol, immersed in xylol, and mounted in balsam.

This clear, differential stain brought out the various stages in the development of the smut-spore walls. A sphere of dark-red material (exospore) formed around the cytoplasmic material, inside the blue-staining,

[1] Hutchinson, W. G. A method for staining rust mycelium in woody tissues. Phytopath. 26: 293–294. 1936.

gelatinous covering of the spore *anlage*. From this sphere, minute brilliant red spines protruded out into the blue-stained jelly of the primitive walls. In shape these spines varied from structures with sharp points to those tipped with small knobs. Many were cone-shape, with considerable variation in length and thickness. In the meantime, the spore itself was growing rapidly in bulk and rounding up; the mature spores with spines being at least 50 per cent larger than the irregular, blue-walled *anlage* derived from the mycelium. In later stages, the blue jelly, which once surrounded the spines and in which they were imbedded, appeared to collapse and disappear. The spines, which stained originally a brilliant red from the orseilline, indicating their lignified nature, changed to a gray, and finally to a brown color, denoting the chemical stages that occurred as they grew and became impregnated with chemicals other than cellulose or lignin (Fig. 1.).

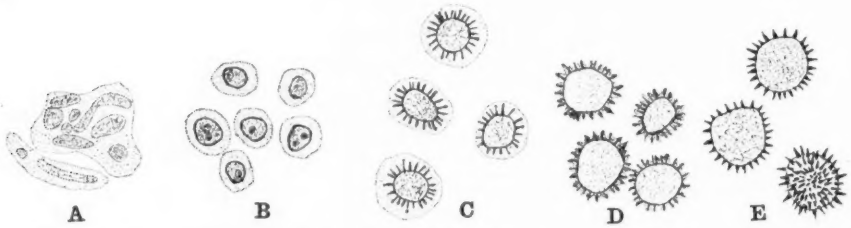

FIG. 1. Spine formation on the spores of *Ustilago zeae*. A. Fragmentation and the rounding up of mycelium with thick, blue, jelly-like walls. B. Inner wall thickens, stains red, as does protoplasmic content. C. Young, red spines protrude from the dark red wall into the blue jelly, which later (D) collapses over the spines. E. A mature spore with gray-brown spines results. Series is magnified about 1000 ×.

Sappin-Trouffy,[2] using methyl blue and either Grenacher's or Boehmer's hematoxylin, but with no details as to methods, was so much fascinated by the binucleate-cell condition in the rusts that he found very little to say about spore-wall development. His accurate drawings, however, indicate clearly that he saw a similar spine formation in the uredio-, aecio- and teliospores as has been just described for smut spores. Wall and spine development are very much easier to follow in the rusts than in the smuts, since the former are so much larger; but the two processes are undoubtedly analagous.

The orseilline BB and aniline blue stain deserves a revival by plant pathologists and mycologists if it is as successful on other fungi as it has proved on smuts and rusts. The majority of the blue stains that the writers and others have tried are very evanescent, but, after 4 months, the sections stained with this combination are as brilliant blue and red as they were the day they were made.—H. L. HUTCHINS and B. F. LUTMAN, University of Vermont, Burlington, Vermont.

The Formation of Chlamydospores of Ustilago crameri Kcke. on Artificial Media.—Numerous chlamydospores were produced in single-chlamydo-

[2] Sappin-Trouffy, P. Recherches histologiques sur la famille des Urédinees. Le Botaniste 5: 59–244. 1896.

spore cultures of *Ustilago crameri* Keke., grown at room temperature for about ten weeks on 1, 2, and 3 per cent potato-dextrose or malt agar in flasks and tubes. Spores were produced equally well on either potato-dextrose or malt agar, but, in general, the cultures produced chlamydospores quicker on the more concentrated media than on the dilute media. These results were obtained in repeated tests and partially corroborate the work of Sartoris[1] with other smuts.

The chlamydospores are formed in an intercalary manner, either from the hyphal strands or from short segments formed by fragmentation of the mycelium, the latter condition occurring infrequently. At the beginning of chlamydospore formation, the mycelial cells, or occasionally alternate cells only, enlarge and become surrounded by a gelatinous membrane. As the spore enlarges still further, the gelatinous membrane apparently is broken and many of the cells disintegrate. There is no wall other than the gelatinous membrane until the spores are two-thirds or more of the mature size. After this a normal episporium and endosporium are formed, the color of the spore gradually darkens, and the gelatinous membrane disappears.

Permanent slides of stained mycelium were made of the cultures in which chlamydospores were forming. Most of the hyphal cells were binucleate, although uninucleate cells were not uncommon, and an occasional multinucleate one could be seen. During gelatinization most of the cells became uninucleate, and fusion of 2 nuclei was observed commonly at this stage.

The chlamydospores produced as described on sterile media are of the same size and structure as those produced in the host plant, and they germinate in a perfectly normal manner.

Several other smut fungi have been reported to produce chlamydospores on artificial media: Among the cereal smuts, *Ustilago tritici*[1] *U. avenae* (see Wang[2]), *U. hordei* (see Kniep[3]), *U. levis*[2], and *U. nuda*[4]; among other smuts *U. tragopogonis* and *U. scorzonorae*[5], *Entyloma ranunculi* and *E. calendulae*[6], *Urocystis anemones*[3], and *U. gladioli*[7].—C. S. Wang, formerly graduate student, Department of Plant Pathology, University of Minnesota; now, College of Agriculture, Honan University, Honan, China.

[1] Sartoris, G. B. Studies in the life history and physiology of certain smuts. Amer. Jour. Bot. **11**: 617–647. 1924.

[2] Wang, D. T. Contribution à l'étude des Ustilaginées (Cytologie du parasite et pathologie de la cellule hôte). Le Botaniste **26**: 539–670. 1934.

[3] Kniep, H. Die Sexualität der niederen Pflanzen 544 pp. (Jena). 1928.

[4] Rodenhiser, H. A. Physiologic specialization in some cereal smuts. Phytopath. **18**: 955–1003. 1928.

[5] Koudelka, H. Neue Probleme in der Brandpilzfrage. Nachr. ü. Schädlingsbekämpf. **9**: 100–104. 1934. [Abstract in Rev. Appl. Myc. **13**: 749. 1934].

[6] Stempell, K. L. Studien über die Entwicklungegeschichte einiger Entyloma-Arten and über die systematische Stellung der Familie der Sporobolomycetes. Zeitschr. Bot. **28**: 225–259. 1935.

[7] Wernham, C. C. Chlamydospore production on artificial media by *Urocystis gladioli*. Phytopath. **28**: 598–600. 1938.

NOTICE TO MEMBERS

Your assistance is again sought in an effort to bring the advantages of membership in our Society to certain others, not yet enrolled, who have an interest in plant diseases. All persons interested in the study of Phytopathology including the practical control of plant diseases shall be eligible to membership.

In addition to active research workers and advanced students such "susceptes" may be found among certain extension pathologists, college and high school teachers, nematologists, entomologists, plant breeders, horticulturists, agronomists, foresters, chemists, officials engaged in plant-disease-control campaigns, in inspection of shipped products, in plant-quarantine enforcement, and in inspection and certification of nursery stock, plants, seeds, etc., county agents, competent persons engaged in manufacture, testing, and sale of fungicides or machinery, and qualified persons engaged in commercial plant-disease control.

It is not the intention that a "high-pressure drive" should be made resulting in the enrollment of a number of people who have no permanent interest in our society, who would soon drop out, and who might do the Society more harm than good. It is, however, the earnest hope that all who would make acceptable members be approached and given a personal invitation to join. Younger men often feel, and rightly, that membership in The American Phytopathological Society indicates an acknowledgment of recognized interest and standing in plant-disease work.

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